

The Gulf War and the NPT

There is a danger that Iraq's supposed nuclear ambitions will undermine the Non-Proliferation Treaty. The United States should formally complain that Iraq is in violation. Then the treaty's well-wishers should make it stronger.

WITH luck, this week should have determined how the war in the Gulf will eventually end. Either the negotiations in Moscow last weekend will have pointed to the basis for a peaceful settlement or the war in the air will have taken to the land and the sea, with the consequent commitment of the armies engaged to termination by defeat. Meanwhile, there is an important issue that must not be hidden by whatever follows. Iraq, a member of the nuclear Non-Proliferation Treaty (NPT), appears for some time to have been in violation of it. Because the treaty is now one of the most generally applicable arms-control agreements (there are 120 signatories), because it serves a useful purpose and because there is a danger that flagrant violation by one member may loosen the adherence of many others, something must be done to strengthen the NPT. But what?

That Iraq may have had ambitions to make nuclear weapons is unremarkable, given the way in which it has accumulated other weapons. But that is not to say that all of Iraq's past interest in nuclear energy is disreputable. In 1977, the Iraqi government began work on a nuclear research establishment at a place called Tamuz, including a research reactor fuelled by a small amount of enriched uranium supplied by France. It was (and is still) reprehensible that Israel, acting on the supposition that the facility was intended for military purposes, should have taken it upon itself to bomb the facility and its reactor in the summer of 1981. In reality, the agreement with France required that the small charge of enriched uranium fuel should be returned to France for reprocessing. After the invasion of Kuwait last August, an inspection by the International Atomic Energy Agency (IAEA) showed the fuel to be intact.

Evidence

So what other evidence is there that Iraq has violated the treaty? The NPT requires of non-nuclear states "not to manufacture or otherwise acquire" nuclear weapons or nuclear explosives and to submit all nuclear facilities and fuel to IAEA safeguards. The matter of the sting operation in which the US Customs last year replaced by duds some 40 capacitors of a kind used in nuclear triggering mechanisms (later "discovered" at London Heathrow Airport on their way to Iraq) is only circumstantial evidence. But towards the end of last month, the military command in Riyadh announced that two nuclear reactors

elsewhere in Iraq had been destroyed early in the conflict. Presumably their identity was based on intelligence data, probably from reconnaissance satellites. That, so far, is all that is certain.

For the sake of the NPT and its continuation, the United States should now formally complain to the IAEA that Iraq is in violation. The matter is too important to give weight to the usual argument that public complaint will also disclose the performance of intelligence gathering. In this case, it would probably suffice that no more detail should be handed over to the board of governors of the IAEA than would be found in photographs from the French SPOT satellite, for example. But to fail to go through the motions specified by the treaty would surely signal to other non-nuclear members than Iraq that running clandestine nuclear explosives plants alongside legitimate (and declared) civil facilities is now permitted. That would mean that the treaty would cease to be an assurance to its signatories that the others also keep to the rules.

London Club

If the United States can sustain its case that Iraq has been in violation, the question will then arise of what penalty should apply. None is specified in the treaty as it stands, which nevertheless allows a member to give three months' notice of withdrawal if it can demonstrate that events with nuclear connotations have "jeopardized its supreme interests". Iraq, no doubt, could have argued at any time in the past 20 years that Israel's emergence as a crypto-nuclear power, or at least its continued refusal to accede to the NPT, provided such an excuse, but Iraq has not used it. Accordingly, the appropriate penalty, within the framework of the treaty, is that Iraq should be denied all technical assistance with nuclear energy, including supplies of equipment and fissile or fissionable material. That argues most immediately for a tightening of the agreement reached in 1977 among the chief suppliers of nuclear technology (the so-called 'London Club'), but the better course would be to amend the NPT itself to require that black sheep, such as Iraq may have been, should suffer from a mandatory embargo in the nuclear field once their status had been established.

Is that feasible? There is at least an opportunity — the conference due to be held in 1995 on the twenty-fifth anniversary of the treaty's entry into force. The snags?



The non-nuclear members of the treaty, already resentful of the dominance of the nuclear powers, will chafe at what seems to be a further assertion of supra-sovereign rights. And that conference is in any case meant to decide whether the NPT should continue in any form. The experience of the past few months has made the NPT seem all the more necessary, but the strains of the same period will probably have further disaffected its non-nuclear members. □

Community confusion

The European Communities have yet to establish a means of supporting research that commands respect.

SIGNOR Filippo Pandolfi, the member of the European Commission responsible for research who is also a vice-president of the Commission, seems bent on dismantling one of the few research initiatives of which the European Communities (EC) can be proud. It now seems that the Commission is planning to scrap the modest programme of grants for basic research, on which it spends about 35 million ECU a year (about £25 million), in favour of an ambitious scheme to scatter graduate fellowships around European universities and research institutes (*Nature* 349, 556; 14 February 1991). Hopes of rescuing some elements of the present research grants scheme (called the SCIENCE programme) rest on tucking them into Pandolfi's plan for graduate fellowships, which entails the choice of 'centres of excellence' throughout Europe. But that solution is at best a second best, and is probably no solution at all.

Most of what the EC spends on research goes on applied research, under schemes for providing 50:50 support for projects in information technology, telecommunications and the like. Collaboration between industrial companies or research institutes in different member states is a prerequisite of support. These projects owe their existence to the mood of the late 1970s, when Europe added to its ambition of achieving industrial parity with the United States the sense of the challenge then presented by Japan. The EC's applied research projects are supposed to be 'pre-competitive' in the sense that they will not lead immediately to saleable products or services, but the definition seems much looser than that used by, for example, the British government for deciding when industry must pay for its own research and development.

Yet these schemes will use up the bulk of the EC's spending under its current framework programme (begun on 1 January 1990), even though circumstances have been radically changed by the prospect of a genuinely free European market from 1 January 1993; that will require industrial companies in different states not to collaborate, but to compete.

That is one reason why there should be a shift in the balance of EC research spending from applied to basic

research. Few now dissent. Another is that many parts of the community are at present ill-equipped to play a full part in an increasingly technical future — countries such as Greece, Ireland and Portugal spend so little, nationally, on research support that few of their young people are likely to join Europe's brave new world. On the face of things, Pandolfi's plan for a huge fellowship programme would help meet that need. By being designated as such, centres of excellence would become more excellent, and in the process would also provide research training for people denied opportunities in their home states. That Europe needs some kind of fellowship programme goes without saying. The puzzle is that, to this end, Pandolfi wants to sacrifice the research grants scheme.

That is a thoroughly eccentric ambition which also overlooks one feature of the research-grants scheme that is at once the explanation of its consistent success and a necessary part of all EC decision-making on basic research: decisions on how the (small) pot of money should be spent have been made directly by a committee (called CODEST) of scientists agonizing about the quality of grant proposals, and not by civil servants balancing scientific advice against other considerations, some political. Throughout Western Europe, evaluation of the CODEST kind is a familiar process, which the scientific community takes seriously. It is no wonder that CODEST has won the most outspoken applause for the work it has supported.

So why end such a success, modest though it may have been? It is understandable that a new research commissioner should wish to make a distinctive mark on the apparatus he inherits, but does that imply that all his predecessors' innovations must be scrapped? In justice, the new framework programme should have financed a substantial increase, perhaps a doubling, of the funds that CODEST has to spend, for there has for a long time been too long a tail of good projects that cannot be supported. Is it pique of some kind, or just indifference, that prompts Pandolfi's determination to head off into the uncharted waters of identifying centres of excellence, and then blessing them with fellowships in their gift? Whatever the explanation, one thing seems certain: there will be a hiatus in EC spending on basic research while the issue is talked out in Brussels. □

Afraid of flying?

One of the greatest benefits of technology — cheap air travel — is at risk from market manipulation.

Mrs Barbara Bush, the US president's wife, did her bit to rescue US airlines from people's fear of flying by buying herself (and nine attendants) seats on a commercial flight from Washington to Milwaukee. But the airlines' problems go much deeper. Pan American Airlines is protected from its creditors only by enlightened bankruptcy

legislation, Transworld Airlines is not much better off, while Eastern Airlines finally went out of business two weeks ago when its equipment was auctioned off at half-a-dozen hub airports in the United States. The standard explanation is usually given as one word: deregulation, which is a reference to the scrapping by the Reagan administration of the practice by which civil servants used to decide which airlines could fly between which pairs of cities and, often, what fares they could charge. Critics of deregulation argue that the outcome has simply been to replace two once-dominant airlines (PanAm and TWA) by two others (United and American). But that is an over-facile and even irrelevant observation.

Elsewhere in the world, airlines are in trouble for reasons that are almost the opposite: too much regulation. Even in Europe and Japan, governments retain a financial stake in (and responsibility for) national airlines, which is a reminder of how an airline and a television station were once the symbols of nationhood. Elsewhere in the world, outright ownership is the rule. And even where deregulation has been given a chance, the old ways persist. The chairman of the Federal Aviation Authority has been musing in public that US legislation might be altered to let foreign interests control up to 49 per cent of the stock of US airlines without explaining why 51 per cent (or even 100 per cent) would be improper. In Britain, meanwhile, the government has declined after nearly three months to decide whether PanAm and TWA could transfer their rights to operate from London's Heathrow Airport (to United and American respectively).

These are disgraceful ways of depriving the world of the benefits of a marvellous technology whose application is hamstrung by three awkward economic characteristics — demand for travel is seasonal, the marginal costs of carrying extra people are low and overall capital costs are high. Most airlines would be more successful than at present if they were truly international, if the regulators allowed them to discount the fares they charge to frequent travellers (instead of to off-peak travellers) and if the regulatory authorities required of them not merely vigilance on safety but on balance sheets as well. (If capital ratios are required of banks, why not of airlines?) Otherwise, if artificial national subsidies were swept away, so might safely be the surviving regulations. □

Indian reputations

The suspension of an infamous Indian palaeontologist does not mean that the rest of India must hang its head.

THERE are two things to say about the decision by the Panjab University of Chandigarh that Professor V. J. Gupta, the palaeontologist, should be suspended from his post (see page 645): first, it is the right decision and, second, it should have been taken a year ago. After all, nearly two years have now passed since professional criticisms of Gupta's published work were given wide

publicity. Even after allowing for the instinctive inclination of academic institutions to defend their favourites, Chandigarh seems to have let loyalty get the better of good judgement by failing to give the allegations against Gupta the weight that they manifestly deserved. Even as recently as last month, it seemed ready to supplement the damning reports of the two inquiries so far undertaken by a third — that of a proposed inquiry by a retired high court judge. Its hand seems to have been forced only by the publication of the gist of the report from the Geological Society of India.

The lesson that the University of Chandigarh should have learned from elsewhere is that allegations of malpractice, while fortunately rare, must be dealt with quickly and decisively. Moreover, there are the strongest reasons why an accused's own institution should take the initiative in the investigation of cogent complaints, however they may arise. Not the least of these is that academic institutions failing to take on such admittedly distasteful tasks hazard their claim to govern themselves autonomously.

But may not the allegations against Gupta, suggesting that he had salted Himalayan strata with often banal fossil specimens from elsewhere, have been a pack of lies? That belief, to which the university has clung for too long, would have been the easier to sustain if Gupta had proved able to respond to the criticism of his work by making his research material available for responsible inspection. Instead, he has blustered that the allegedly misattributed fossils were indeed found in the strata in which their discovery was originally reported (see *Nature* 338, 613; 1989). That does not in itself imply that Gupta's position is untenable, but it did require that the university should have taken steps to see that Gupta's fossils were made accessible — and that it should have suspended him if he disagreed.

What does all this imply for the reputation of Indian science? Is it all tarred with the same brush? Many in India fear that people elsewhere will jump to that conclusion, but it would be incorrect. As the world knows, much research in India is excellent by accepted international standards. As everywhere, much of it is also humdrum, but honest. India's difficulty is that it is a society in rapid transition, where the traditions of what constitutes proof of discovery developed since the Second World War coexist with those from earlier times. Some institutions have not yet shaken off the notion that the director of a laboratory or the head of a department is by definition a big shot in every way, entitled to keep his junior colleagues in thrall. (This journal will make it its business to see what happens now to Gupta's ex-colleagues at Chandigarh, and will protest if they are victimized.) There is also a somewhat mystical hankering after theories of everything. But there is no reason why people elsewhere should be misled by these circumstances, but, rather, every reason why they should, with discrimination, honour good Indian science for what it is — an important contribution to all our understanding. □

DNA research institute to open in Japan

- Support from local government and industry
- Is this Japan's real human genome project?

Tokyo

AT a time when Japanese scientists are struggling to win significant central government backing for a human genome project, a small local government with the backing of private industry has made the first move. Chiba prefecture, a region that lies on the opposite side of Tokyo Bay from the capital, has just announced plans to build a DNA research institute in a science park that will open in 1993.

Although the institute in Chiba will get no funds from the federal government, it nonetheless has won an official stamp of approval from the federal Ministry of International Trade and Industry (MITI).

Although no one knows exactly how the new institute will fit into government and international plans to sequence the human genome, one of its main functions seems likely to be the actual sequencing of genes, rather than work on related basic research on the human genome. The sequencing job is more a technical than a scientific challenge, and the Chiba institute seems intent on conquering it.

One Japanese scientific adviser to the institute says that there is a close similarity between this institute and a proposal by Akiyoshi Wada, formerly of Tokyo University, to establish a DNA sequencing 'factory' in Japan. Wada's philosophy is that DNA sequencing is not an appropriate activity for scientists but rather is something to be carried out by technicians and machines. Comments from various advisers to the project indicate that the prime role of the Chiba institute will be to provide a non-profit sequencing service for Japanese academics.

What does this imply for the various human genome projects under the administration of the Ministry of Education, Science and Culture, the Science and Technology Agency and the Ministry of Health and Welfare? The striking thing about the Chiba institute is that many of its team of academic advisers also serve as advisers for the small government projects (although it is perhaps not so surprising considering the limited number of distinguished academics involved in this field). One adviser suggests that the Chiba institute will turn out to be the focus of the hard effort and that bright Japanese academics voice support for the other projects simply to draw as much money as possible out of the central government, which is very short of funds

because of enormous debts built up during large construction projects in the 1970s, such as the building of bullet train lines.

But while the central government is hungry for cash, local governments have more money on their hands than they know what to do with. The Chiba government, like several other local governments, has decided to invest its surplus cash in a science park and, in particular, DNA research. The Kazusa DNA research institute will be the first institute to open in the Kazusa Academia Park, a collection of largely private-sector research institutes being planned by the Chiba government (see adjacent story).

Susumu Tonegawa of the US Massachusetts Institute of Technology (MIT), who won the 1987 Nobel prize for medicine, and his mentor Itaru Watanabe, vice-president of the Science Council of Japan, were very influential in persuading the Chiba government to establish the new institute. With an initial fund of ¥5,000 million (\$40 million), 75 per cent of which will be provided by the local government and most of the rest by industry, the institute will start with a staff of about 70 in 1993, increasing to around 100 once the institute gets into full swing.

Among the private companies that will lend support are Nippon Steel Corporation, Kawasaki Steel Corporation, Tokyo Electric Power Company, Tokyo Gas Company, Hitachi, Mitsui Toatsu Chemicals and several local banks, according to Kenji Goto, deputy director of MITI's office for the Human Frontier Science Programme. MITI will also lend its support by giving an official stamp of approval to the foundation that will back the institute. No central government funding is expected.

According to Chiba government officials, the institute will focus on sequencing important human genes as well as on developing computer systems and software for the analysis of DNA. But, in an apparent contradiction, they say that the institute is "unrelated" to international and domestic government-backed efforts to sequence the human genome. Their reluctance to admit the obvious connection may be political, and is the sort of thing that can be done only in Japan where, provided one takes an acceptable public stance, nobody will interrogate one about one's true intentions.

David Swinbanks

SCIENCE CITIES

From parkland to science park

Tokyo

AT present, the Kazusa Academia Park consists only of tree-covered hills on the opposite side of Tokyo Bay from the capital. But the recent success of Tsukuba science city has inspired Chiba prefecture, where Kazusa will be built, to invest in a high-technology future. By the end of the decade, its backers expect Kazusa to be a small but bustling city that is home not only to the planned DNA research institute but also to a number of industrial research centres for such companies as Fujitsu and Canon.

Ten years ago, the success of science cities seemed far from guaranteed. Tsukuba, located north of Tokyo, had been established by the central government in the 1970s, but the 'city' lacked adequate shops, schools or transportation.

Since the mid-1980s, however, Tsukuba has blossomed. The Tsukuba Science Expo in 1985 brought in hotels, department stores and restaurants, and the past five years have seen an influx of dozens of private company research institutes (see *Nature* 345, 378; 1990).

Now, building science cities and science parks has become all the rage among local governments in Japan, which have far more cash than the nearly bankrupt central government. The Kazusa Academia Park is just one of more than a hundred high-tech centres planned or already under construction, including about ten research cities.

Kazusa will be smaller than the Tsukuba and Kansai science cities, with a planned area of 1,000 hectares (about one-third the size of Tsukuba). While officials of the Chiba Prefecture point to Tsukuba as their source of inspiration, they say they hope to learn from the mistakes there and will concentrate on attracting private sector institutes to the park.

They have already had success in attracting industrial research centres, both large and small. Fujitsu plans a 7,500-person institute that will focus on electronic devices and communications. Ajinomoto, Japan's largest manufacturer of food additives and amino acids, will install 90 researchers to investigate protein and genetic engineering. Other companies that have decided to establish institutes in the park include Canon and Nissan Chemical Industries.

Although Kazusa is isolated now, that is changing. A huge Tokyo Bay Bridge will link the park to Tokyo when the bridge is completed in 1995, and a loop road around the city will bring the park within easy driving distance of Tsukuba science city and Narita international airport.

David Swinbanks

Rules arrive under a cloud

Washington

Six years after Congress passed controversial legislation requiring strict new rules for handling laboratory animals, the US government has finally finished those rules, if not the controversy.

Last week, the US Department of Agriculture (USDA) released an 85,000-word final regulation for the treatment of research dogs, cats and non-human primates. The new rules follow two previously published sets of regulations on smaller animals and finish what has essentially been a three-part rewriting of the entire Animal Welfare Act.

Years of debate over the need for stronger regulations and the changes that are appropriate led Congress in 1985 to pass amendments to the Act. But legislators left unclear exactly how they wanted USDA to rewrite the rules. So the past six years have seen a pitched battle between researchers and animal-welfare activists to determine the final form of the regulations, a struggle the research community now seems to have won.

The new regulations set specific standards for such concrete matters as cage sizes but leave the particulars of animal exercise and psychological health largely in the hands of the experimenters — an arrangement that researchers applaud. Animal-welfare activists, however, who had originally fought for the regulations and then sued to speed their release, attacked the rules as a “great leap backwards” and threatened further law-suits to force USDA to set more rigid standards.

The disagreement centres around ‘engineering’ versus ‘performance-based’ standards. In matters such as cage sizes and environmental conditions such as temperature and humidity, the new regulations essentially codify the existing housing and treatment guidelines of the National Institutes of Health (NIH) by setting minimum dimensions and enclosure volumes. But in matters of animal exercise and ‘lifestyle’, USDA has adopted performance-based standards.

Agency inspectors will observe the animals to determine if they act in a healthy and well adjusted way, rather than to prescribe exact minimum procedures and cage configurations. Researchers will have to document that the procedures they use have been shown to encourage healthy animal behaviour. Should a USDA inspector find evidence of psychological abnormality in a primate, the burden of proof will be on the institution to show that the problem is not a result of inappropriate housing conditions.

Even in the case of enclosure dimensions — normally a strictly engineering matter — USDA will now permit some performance-based flexibility. The

new rules permit ‘innovative caging’: housing that may not exactly fit the cage size formula, but nevertheless allows for healthy animal behaviour.

Although the scientific community has historically opposed more regulation of animal facilities, research advocates have praised the new rules. By adopting the NIH guidelines, USDA has increased minimum cage sizes in many cases. But most research facilities, as recipients of NIH grants, had already adopted the more stringent standards, says Barbara Rich of the National Association for Biomedical Research. “For most institutions, probably the majority, the new rules will mean no change”, she says.

With regret, animal-welfare activists agree. Congress had intended vast reform when it passed the amendments to the Animal Welfare Act in 1985, they argue. But if research facilities find that the new regulations require little change, animal welfare can hardly be expected to substantially improve as a result. Performance-based rules, says William Cotreau of the Animal Welfare Institute (AWI), “are like no rules at all. They’re suggestions.”

The San Francisco-based Animal Legal Defense Fund (ALDF) intends to file a suit charging USDA with violating congressional intent, but even activists admit that the congressional language on the amendments is unclear. And, as USDA points out, Congress’s 1991 agriculture appropriations bill calls for “performance-based standards . . . when such standards would not interfere with the establishment of a minimal level of care or the enforceability of the Act”.

UK RESEARCH

British scientists hit back

London

An open letter to the British Prime Minister, John Major, signed by 72 distinguished expatriate British scientists, emphasizes the critical condition of British science and demands government action to reverse the trend. The letter, organized by the California-based pressure group British Scientists Abroad (BSA), comes almost exactly a year after a BSA petition with 1,647 expatriate signatories was snubbed by the government.

That earlier petition (see *Nature* 343, 499; 1990) had been organized in response to government statements that the so-called ‘brain drain’ did not exist, because researchers from abroad came to Britain to fill the gaps left by those leaving. The petition called on the government to increase research support in line with civil research expenditure in other major industrialized nations, and to institute tax

“Overall, we believe we produced a practical and enforceable blend of performance and engineering standards”, said James Glosser, administrator of USDA’s Animal and Plant Health Inspection Service, in a prepared statement last week. “We have made room for variations in the way individual animals behave and for differences in the way they are used and housed. We also have provided flexibility in complying with the regulations by offering more than one option for achieving certain standards.”

USDA estimates that compliance with the new rules will cost US researchers \$537 million. A stricter version of the rules more in line with the demands of the animal-welfare activists would have cost research facilities \$1,750 million, or more than three times as much, USDA calculated. The shift to performance-based standards was in large part due to what USDA considered an unreasonable expense to the research community.

AWI, however, counters with its own figures. An analysis of the USDA calculations released recently by AWI claims that accounting errors and erroneous assumptions overstate the cost of the more strict rules by over \$1,000 million. With the new accounting, AWI argues that the strict rules would cost little more than the regulations USDA actually released, while significantly improving the lot of research animals. Should ALDF file suit as expected, it will probably raise that issue as well.

But unless a federal judge rules against USDA — an outcome that would be rare in such a case — the new rules will stand and the battle that has clouded the Animal Welfare Act for six years will be over.

Christopher Anderson

incentives for industrial laboratories that publish their research.

Although 24 of the signatories of the original petition were fellows of the Royal Society and more than a hundred were heads of department, the government dismissed it as unimportant on the grounds that most of the signatories held junior positions. This excuse may be undermined by the list of signatories of the new open letter, which reads like a roll-call of illustrious innovation. They are all fellows of either the Royal Society or the Fellowship of Engineering, with addresses predominantly in the United States.

The open letter restates the message in last year’s petition, adding that “unless the government acts to halt the decline in research, we fear that Britain will become a minor player in technological development. The consequent economic and social damage would be severe.” **Henry Gee**

Presidential address sets the tone

Washington

THE annual meeting of the American Association for the Advancement of Science (AAAS) captured the attention of some 4,700 scientists in Washington this past week. The fare at the AAAS's 157th annual meeting included science as well as policy, light items as well as more serious research.

It may well be the last year, however, that the AAAS keeps this format for its annual meeting. At a time when specialized meetings seem to dominate the scientific landscape, the AAAS is thinking about revamping its meeting programmes. Declining attendance over the years has made some AAAS leaders wonder whether the multi-disciplinary annual meeting should be continued.

A brief speech by US President George Bush opened the five-day meeting.

■ Bush speaks to AAAS

ON the morning of Friday 15 February, an estimated 3,000 scientists gathered in a hotel ballroom in Washington to hear President George Bush address the AAAS meeting. But the news of the moment was not science but war. Only hours before, word had come from Baghdad that the Iraqi leader, Saddam Hussein, would pull his troops out of Kuwait. Many people expected that Bush would cancel his AAAS speech, which was to be broadcast by closed-circuit television because the secret service forbade a personal appearance.

But no. The president kept his promise to speak to the scientists, seizing the occasion to deliver an address on the politics of war and of science.

Acknowledging that when he first heard the news from Baghdad he was "happy", Bush went on to denounce Saddam's offer to withdraw as a "cruel hoax". Upon analysis, the president said, the Iraqi statement was "full of unacceptable old conditions", and unacceptable new conditions as well. "Let me state once again", said Bush, "that they must withdraw without condition." It was, as far as anyone could remember, the first time that a US president has delivered such a potent political statement to an audience of scientists.

But it was not the first time this president has symbolically acknowledged the scientific community with a direct appearance. Last spring, Bush spoke at the annual meeting of the National Academy of Sciences. His AAAS speech is seen as a reiteration of his recognition of science, as well as testimony to the influence of his own science adviser, D. Allan Bromley, within the White House.

When he reached what he called "the subject at hand", Bush touched on all the

themes on the current science agenda, including the success of past investments in military research and development, a theme that is likely to dominate US debate on the future budget for the Pentagon. "Technology", he said, "is changing the face of war." Citing the Patriot missile in particular, Bush said "Our investment in defense [R&D] over the past decade is now saving the lives of Americans, of our allies, and even of our adversaries." The president spoke of the importance of science and mathematics education in the schools and quoted Nobel laureate Barbara McClintock who said of her work on the genetics of corn, "I did it because it was fun. I never thought of it as science." The president sought credit as a 'science' president for the 13 per cent increase in funding he requested in the 1992 budget, he cited the importance of the "individual scientist" or "small team", but he also gave the nod to such big science efforts as high-performance computing and communications, the human genome project and the Global Change Program.

The president saved his most original observations, however, for the subject of science in industry (he called for a permanent R&D tax credit) and innovation. He declared that "the spirit of innovation is alive and well in America", and touched on the issue of what industry considers excessive or stifling government regulation. "Some say that if Edison had invented the light bulb today, we'd have scores of studies citing the dangers of electricity. And the newspapers would headline the story 'Candle industry threatened'."

■ Meta-analysing the risks

THE epidemiological evidence linking electromagnetic fields (EMFs) with health effects, such as cancer and birth defects, has generally been suggestive but quite weak. Several studies have found, for instance, that living near electric power lines of a certain type may increase the risk of leukaemia and other cancers in children, but other studies have seen no such effects. Lacking any laboratory evidence of such an EMF-cancer link, scientists have been divided on whether EMFs might be dangerous.

At the AAAS meeting, Edward Washburn of the Harvard School of Public Health released the results of a new study indicating that the presence of the fields may indeed signal increased risk. A meta-analysis of existing epidemiological studies found, Washburn said, that living near certain power lines may double a person's chance of developing central nervous system cancers and increase the risk of lymphoma by 50 per cent.

Meta-analysis is a technique for integrating the results from many different

and even contradictory studies on a single subject. Although controversial, meta-analysis has become increasingly popular over the past 15 years as an objective way of extracting a consensus answer from a mass of inconsistent data. Meta-analyses have been performed on subjects as varied as gender differences and the effectiveness of psychotherapy, but probably the most popular area for meta-analysis has been the health effects of various environmental and lifestyle factors.

For instance, Judson Wells of Kennett Square, Pennsylvania, described to the audience at the AAAS session his 1988 meta-analysis of passive smoking studies. Working with the data from 17 passive smoking studies around the world, he found that non-smoking women who lived with smokers were nearly 50 per cent more likely to contract lung cancer than non-smoking women who were not exposed to smoke in their homes. William Nicholson at the Mount Sinai School of Medicine in New York City discussed his meta-analysis of the effects of exposure to polychlorinated biphenyls (PCBs) on workers who came in contact with the chemicals in capacitor manufacturing plants. Analysing the various studies with respect to the subjects' durations of exposure and the times from onset of exposure, he found evidence that PCB exposure causes cancer of the liver, biliary tract and gall bladder.

Washburn and colleagues at Harvard, Mount Sinai and the University of Pennsylvania are in the midst of a series of meta-analyses examining the contradictory results from studies on the health effects of low-frequency EMFs, such as those generated by power lines and electrical appliances. At the AAAS meeting, he revealed preliminary results from a meta-analysis of ten studies on the correlation between proximity to power stations and the risk of leukaemia, lymphoma and central nervous system cancer in children.

The researchers went to great pains, Washburn said, to make sure their analysis was as reliable as possible, given that they were working only with existing studies and took no data themselves. They rated the quality of each study, using referees who did not know which study was which; they tried to make the assessment of EMF exposure consistent from study to study; and they searched the literature in order to include all studies on the subject.

The result? They found that residential proximity to power stations was linked to a higher risk of cancer in every category they examined, although the results were statistically significant in only two cases. The calculated risk ratio for central ner-

vous system cancer was 2.01 — meaning that exposed children were 2.01 times as likely as unexposed to develop cancer — with a 95 per cent confidence interval of [1.44, 2.82]. (That is, although the 'true' risk ratio is not known, it is 95 per cent certain that it falls between 1.44 and 2.82.) The risk ratio for lymphoma was 1.52, with a 95 per cent confidence interval of [1.08, 2.12].

Washburn's group also calculated risk ratios of 1.51 for childhood lymphoma, 1.20 for leukaemia and 1.26 for childhood leukaemia, although none of these were statistically significant.

These results slightly strengthen the case for an EMF-cancer connection, but they still suffer from the weaknesses of all of the epidemiological data so far. Researchers have not been able completely to rule out the presence of 'confounders' — factors that appear concurrently with the EMF exposure and that may themselves cause cancer, such as carcinogenic pesticides used to clear the right of way underneath power lines. And, for some mysterious reason, the cancer risk seems to correlate better with the types of power

lines than with the actual measured EMF fields inside the subjects' homes (see *Nature* 349, 554; 14 February 1991). The meta-analyses can point to these weaknesses in the original studies, but they cannot fix them.

■ Don't trust this headline

DECEPTION is rife in the world. Men lie to women, and vice versa. Predators try to fool their prey, and the prey return the favour. Even bacteria and viruses get into the act, with elaborate molecular mechanisms designed to distract, confuse and deceive the immune systems of their unwilling hosts.

Why all the deception? The short answer is that, in an evolutionary sense, it works. The long answer was the subject of a AAAS session on "The evolution of deception: A biocultural approach".

"We are not defining deception as a conscious, nefarious act", said session organizer Loyal Rue, a professor of religion and philosophy at Luther College in Decorah, Iowa. And given that "nature provides niches for deceivers at every level of life", he argued that it might be

necessary to rethink our natural bias against it — even in some cases of human deception.

Examples of deception in nature are common. Viceroy butterflies, which are a tasty treat for birds, mimic the markings of the foul-tasting Monarch butterfly to trick birds into not eating them. Some male blue-gill sunfish mimic both the appearance and the courting behaviour of females so that they can enter the territories of dominant males and fertilize the eggs laid there by the females. Cuckoos fool other birds by laying eggs in their nests. All of these tactics provide some evolutionary advantage and so developed through natural selection.

The more complex deceptive practices, however, are the behavioural ones, especially those that are learned instead of innate. Robert Sussman, an anthropologist at Washington University in St Louis, described deceptive behaviour in primates which, he said, seem to be the only animals that engage in 'voluntary deception'. He described one experiment in which a single female chimpanzee was shown the location of food in a cage. When the female and other members of her group were let into the cage, she went excitedly to the food — at which point the other chimpanzees took it away from her. After a few repetitions, she got the hint. When next the group was let into the cage, the female did nothing at first. Then, once the others were all settled down and looking the other way, she grabbed the food for herself. "Deception", Sussman said, "seems to have evolved along with intelligence." That leaves humans in the position of being the earthly creatures most able to deceive others, as well as themselves. How humans have dealt with that ability is one of the fundamental issues underlying Western religion and philosophy, Rue said.

Despite the presumption that deception is wrong, the ability to deceive and to be deceived seem to be adaptive traits, he noted. The value of being able to lie convincingly is obvious, but being vulnerable to deception, particularly self-deception, can also be valuable. Studies have shown, for instance, that people with positive self-images — including those that involve a certain amount of self-deception — are usually happier and healthier than those with low self-esteem.

The advantage of deception does not stop with individuals, Rue argued. The health and even existence of human cultures has historically depended on what he calls 'noble lies' — myths, or self-deceptions, that are shared among the people of a culture and which define a common way of viewing the world. As long as the vision is left unexamined, it can provide a framework on which to build a society.

Barbara J. Culliton and Robert Pool

Hope for the musically mediocre?

FOR everyone who dreams of making beautiful music but whose fingers just cannot seem to find the right notes, there is hope. In a presentation at the AAAS meeting, Max Mathews of the department of music at Stanford University described a new computerized system that gives a person creative control over music without the necessity of first perfecting his or her technique.

A musical performance, Mathews explained, consists of two parts: the score, a predetermined sequence of notes fixed by the composer; and the expressive factors, such as tempo, subtle changes in the duration of notes and the loudness or softness of different passages, which the individual performer controls. But the score provides a difficult hurdle for some would-be performers because it takes many tedious years of practice to develop the necessary manual skills to reproduce the notes as they were written.

"We can remove this load from the performer by letting the computer provide the correct sequence of pitches", Mathews said, "but at the same time we zealously guard the expressive parts of the music and give complete control to the performer." This is possible, he said, through the use of a computer system consisting of software called a conductor program and a piece of hardware called a 'radio baton'. The conductor program 'reads' a score and instructs a synthesizer to play the correct notes, but the performance is controlled by a human performer wielding the radio baton. This is a stick-like device with a radio transmitter at one

end, which is waved over a detecting surface. The surface detects the radio waves emitted by the baton, determines the baton's position in three dimensions to within a fraction of an inch and directs the conductor program to modify the music in response to the movement of the baton.

The performer controls the tempo of the synthesizer-generated music through the beats of his baton, much as a conductor directs the tempo of an orchestra.

And by varying the position of the baton over the detecting surface, the performer can modify the quality of the notes. The effect, Mathews said, is similar to that which a violinist achieves with his bow or a woodwind player with his breath.

To demonstrate the effectiveness of the device, Mathews played a tape recording of a Vivaldi concerto in which two human flautists were accompanied by a synthesized orchestra which Mathews had directed with the radio baton. To an untutored ear, the sound was practically indistinguishable from a recording of a full human orchestra.

This device could open the world of music to a new type of performer, Mathews said — one who does not have to divide his energy and attention between technical performance and the expressive factors. Mathews admitted, however, that he does not know how good these performers are likely to be. "Can we teach the expressive facilities quickly and simply with this new instrument? Or does a performer need the years of practice on technique [before he can develop these expressive powers]?"

R.P.

Political fallout anticipated

Tokyo

As details of the emergency shutdown of the number two reactor of the Mihama nuclear power plant begin to leak out, it is becoming clear that Mihama is the most serious accident ever in the history of Japanese nuclear power. Although there were only small releases of radioactivity into the local environment, the political fallout from the accident is likely to be much more serious.

Inspection of the primary cooling system with a fibroscope has revealed that one of the thin tubes in the heat exchanger of the primary system was blown open, and rapid leakage of primary coolant into the secondary cooling system activated the emergency cooling system. A similar accident occurred at the North Anna plant in Virginia in the United States in 1987, but Japanese government and nuclear industry officials, who pride themselves on their strict periodic safety checks of nuclear plants, had claimed that such an accident could never occur in Japan. The pipe at Mihama that broke open had passed such a safety check with flying colours last July.

According to the latest reports, when the pipe blew, 50 to 100 tons of radioactive steam poured into the secondary cooling system which drives the plant's turbine —

PR blunders

THE day after the Mihama accident, about 30,000 local residents in the area surrounding the plant opened their newspapers to find a public-relations pamphlet from Kansai Electric Power Company proclaiming the wonders and safety of nuclear power, according to a Kyodo wire service report. Apparently the company, which operates the Mihama plant, failed to withdraw the leaflet because its employees were too busy dealing with the accident.

In describing the cooling system of nuclear power plants, the pamphlet claimed that "high performance" robots can "detect even pinholes that human eyes cannot see", thereby allowing pre-emptive repairs to be carried out. It was a gaping hole in the primary cooling system that caused the Mihama accident.

And things became even more embarrassing for the hapless power company. Mihama plant holds regular tours of its facility for the public. At the time of the accident, ninety-four such visitors were inside the plant, and some reported seeing steam emanating from the turbine room as the reactor was shut down. Fortunately, the visitors were unaware that a major accident was in progress.

D.S.

much more than the 20 tons initially estimated (see *Nature* 349, 557; 1991). And contrary to early official announcements that no radioactivity was released into the local environment, it has now been revealed that radioactive steam was vented from the secondary system into the atmosphere and sea when the primary system blew. But the small amounts of radioactivity went undetected by monitors surrounding the plant, which continued to show background radiation levels.

The timing of the accident was "very bad", say officials of the Ministry of International Trade and Industry (MITI), the government body in charge of the nuclear power industry. Public fears of nuclear power had begun to calm down after the Chernobyl accident, and a pro-nuclear candidate was elected recently as governor in Aomori prefecture where government and industry are establishing a huge facility for enrichment and reprocessing of nuclear fuel and storage of nuclear waste (see *Nature* 349, 449; 1991).

Several days after the accident, government and industry officials revealed that a safety valve designed to relieve pressure in the primary cooling system in case of leaks failed to function. Plant operators had to use a backup system that sprays water into the primary coolant to relieve pressure. As a result, the release of radioactive steam from the secondary system into the environment was larger than if the valve had worked.

Ryuji Fuji, director for international affairs on nuclear power safety in MITI's Agency of Natural Resources and Energy, says that MITI did not immediately announce the failure of the valve because its prime concern was to reassure the public that the local environment had not been seriously contaminated with radiation. But the delay runs counter to government pronouncements that it wants to win public understanding through open and scientific explanation of the safety of nuclear power.

Haruo Suzuki, director of the office of nuclear safety policy research at the Science and Technology Agency, says that from the political point of view the accident is "very severe". For the past few years, the government and the nuclear power industry have been spending millions of dollars on public relations campaigns designed to reassure the public about the safety of Japanese nuclear power in the wake of Chernobyl. Mihama has blown a gaping hole in all those efforts.

In addition to a mandatory investigation by the Nuclear Safety Commission, Prime Minister Toshiki Kaifu has called for a cabinet-level investigation of the accident, and MITI will establish a com-

The Lords weigh in

London

THE influential House of Lords Science and Technology Committee has launched an inquiry into the 1991-92 UK science budget, an action that could ultimately lead to pressure on the government to change its science funding policies. The recently announced science budget precipitated a funding crisis in the research councils, culminating in a savage package of cuts by the Science and Engineering Research Council (SERC; see *Nature* 349, 551; 14 February 1991).

The committee aims to discover whether the research councils' difficulties are primarily the result of the government's failure to increase funding to match inflation, of increasing subscriptions to international science projects, or poor financial management by the research councils themselves. Speaking in the House of Commons on 6 February, Education and Science Secretary Kenneth Clarke laid the blame for the current round of cuts at the door of SERC under its previous chairman, Sir William Mitchell, who stepped down last autumn. SERC had embarked on a series of large commitments that outran its resources, he said.

Unusually, the new inquiry will be conducted by the full 16-member Science and Technology Committee, rather than a small subcommittee. The inquiry's findings will be published in a short report in the coming months. Although the government is not obliged to act on the committee's recommendations, pressure from the committee has influenced UK science policy in the past. The committee will meet Sir Mark Richmond, SERC chairman, and Sir Peter Swinnerton-Dyer, chief executive of the Universities Funding Council, next week.

Peter Aldhous

mittee of 20 experts to look into the affair.

Japan has 17 pressurized water reactors (PWRs) like Mihama currently in operation. Of these, four have had more than 10 per cent of the thousands of thin primary tubes in their heat exchanger plugged because of leaks, according to Fuji. This compares with only 6 per cent plugging in the Mihama No. 2 reactor, which blew.

The government has no plans to close these other plants for inspection. Instead, MITI has simply advised the power companies to make their periodic inspections more stringent.

But the most serious potential fallout from the accident concerns MITI's ambitious plans to build 40 more nuclear power plants in Japan by 2010. Before the accident, the government was already having difficulty winning public acceptance of proposed sites, and Mihama will make siting even more difficult.

David Swinbanks

Gupta faces suspension

New Delhi

PANJAB University geologist Dr Vishwa jit Gupta has been suspended from his posts at the university at Chandigarh in the wake of two reports supporting allegations that his Himalayan fossil collections, the source of the numerous publications spanning two decades, were faked.

Apart from being a professor at the university, Gupta was until his suspension director of the Institute of Palaeontology, supported by the University Grants Committee, and a deputy to the vice-chancellor. He is now being served with a charge-sheet, bringing to an end a two-year controversy triggered by allegations by Dr John Talent (see *Nature* **338**, 613–615; 1989 and see also **343**, 305–308; 1990 and references therein).

Orders suspending the professor were issued last week by the vice-chancellor of the university, Dr R. P. Bambah, acting on reports of investigations by the Geological Survey of India (GSI) and the Society for Scientific Values (SSV), an independent body of scientists concerned with ethics and morality in scientific research. The two reports concurred in their conclusion that Gupta's Himalayan fossils were not genuine. Gupta was not available for comment.

SSV's report was based on an analysis of samples its team brought back from an expedition to the Himalayas in August 1990. The report was not made public, but was sent only to Bambah with a recommendation for appropriate action against Gupta.

The GSI council, on the other hand, decided to give Gupta a last opportunity to come clean, and sent its report to him with a request to "make his collections available and also provide full details of fossil localities and stratigraphic horizons for independent check". When Gupta failed to respond, GSI published the report in its official journal with a leading article that says: "It is obvious from the volume of evidence that has now been collected that the fossil finds of Dr Gupta are not reliable, that there are internal inconsistencies, that the data is incomplete bordering on disinformation."

The GSI, which scrutinized all of Gupta's papers published between 1969 and 1988, said his work was "fictitious and based on spurious fossils", lending support to accusations of questionable practices such as recycling of fossils, plagiarism and scientific misconduct.

Checks by GSI officials and some of Gupta's own colleagues revealed neither the fossils nor the rock formations said to have been present in the areas from which fossils were supposed to have been collected. According to the report, Gupta's replies to the charges were evas-

ive. "He failed to produce originals of fossils with their registration numbers, dates of collection, field notebooks, laboratory registers and other evidences to confirm the genuineness of his collections."

Both SSV and GSI reports were available to Bambah last December, but

HIGH-PERFORMANCE COMPUTING

A last chance to catch up?

London

IN order to compete with the United States and Japan in high-performance computing, Europe should increase its annual investment in the area by 1,000 million ECU (European Currency Units, about £700 million) by 1995. That is the conclusion of an expert working group of European supercomputer users chaired by Carlo Rubbia, director-general of the CERN European particle physics centre.

As noted in the committee's report, almost all high-performance computers are now manufactured in either the United States or Japan, even though European users account for some 30 per cent of the world market. And the next generation of high-speed computer is under development. Machines capable of one million million operations per second, almost a thousand times faster than today's best supercomputers, may be possible by the turn of the century.

The working group, which was set up by the European Commission in 1990, sees this next generation as a window of opportunity for Europe. The report of the working group, however, left many questions unanswered. It did not detail exactly who is likely to pay for the programme or even how the burden should be shared between the public and private sectors. And just how the group arrived at 1,000 million ECU per year as an appropriate increase — which the report characterizes as an "order of magnitude" figure — is equally unclear.

Group members were last week unable to say how much Europe currently spends on its high-performance computing industry. Instead, Commission officials say they were influenced by the level of new investment already planned in the United States. President George Bush's science budget request to Congress for 1992 proposes a 30 per cent increase in high-performance computing research funding, up to \$638 million, as part of an initiative that will also include private-sector spending.

Some critics question whether a European programme in high-speed computing would have tangible economic returns and suggest that the request for massive supercomputing investment is motivated more by questions of prestige.

Gupta's well-wishers wanted the matter referred to a retired judge of the high court. As recently as the beginning of this month, the university was making arrangements for this further inquiry.

But Bambah has now ordered Gupta's suspension because, as one faculty member remarked, "the episode has brought disgrace to the Indian science community".

K. S. Jayaraman

Martin Campbell-Kelly, a historian of the computer industry at the University of Warwick, says that there is no convincing evidence that investment in supercomputer manufacturing and research produces strong "trickle-down benefits" for the computer industry as a whole. He believes the ailing European industry would benefit more from investment in the development of smaller machines, where the potential market is larger.

Apart from the massive US government investment in high-performance computing companies from the 1950s, to provide computing power for the US atomic weapons programme, US companies win over their European counterparts in having a large single domestic market to exploit. Bill Trebinski, marketing manager for the British company Active Memory Technology (AMT), the leading European manufacturer of massively parallel supercomputers, says his company finds it easier to sell in the United States than across Europe, where each country must be treated as a separate small market. But there is also a "psychological problem", Trebinski says, in that both US and European supercomputer users assume US machines are more advanced. AMT, which earns more than 60 per cent of its revenue in the United States, is often thought of as a US company, Trebinski says.

The forthcoming 'single European market', due in 1992, may break down many of the trading barriers that restrict the European high-performance computing industry. But Tor Bloch, a member of the Rubbia working group, says Europe must also work to create a "common culture" for its supercomputer users, if the single market is to become a reality in high-performance computing.

Europe needs "the computing equivalent of CERN", says Bloch, whose own organization, the Advanced Computing Research Institute of Lyon in France, is now assembling an international team to design new high-speed computers.

To this end, the Rubbia group's report says that 200 million ECU of the 1,000 million ECU a year is needed to establish high-speed electronic networking between supercomputers and service centres throughout Europe.

Peter Aldous

Problems of funding research

SIR—The current problems in funding of academic research by the Science and Engineering Research Council (SERC) and the Medical Research Council (MRC) contradict the British government's stated policy of "level funding" for scientific research. The present crisis seems to derive from underfunding of salary increases for SERC and MRC staff, and the resulting shortfall being made up from the money allocated to project grants.

Industry has attempted to carry an ever-increasing proportion of biomedical research support in Britain over the past five years, as evidenced by large grants by companies to university and polytechnic departments. Industry funding in support of LINK schemes, research councils, cooperative awards, post-graduate support work and general support for research projects is of the order of £100 million annually. This compares favourably with the total research councils' budget.

As scientists working in the pharma-

ceutical industry, we are concerned about the damage that limitation of funding is already causing. As it is, the research councils do not have sufficient funds to support all alpha-rated research and they now seem unlikely to be able to fund any significant new scientific initiatives during the coming year. This does not provide an atmosphere that encourages young scientists to enter research as a career, particularly in the academic sector. The UK science base must inevitably be eroded in the longer term as a result.

British academic and industrial scientists have an enviable record of scientific innovation. Three of the five most successful prescription medicines introduced worldwide in recent years were discovered in Britain. Although industry will continue to support and collaborate actively with academic science departments, we believe it is the responsibility of government to provide an adequate infrastructure to maintain our successful research base, both academic and industrial. The

British pharmaceutical industry made a positive contribution to the balance of payments of almost £1,000 million in 1989 and it has been one of the United Kingdom's major export earners for many years.

Failure to provide academic research support will drastically limit the numbers and scope of the researchers and teachers of tomorrow as well as restricting the growth (or even maintenance) of our successful industry. We hope that the government will recognize the importance of its role in the support of science in the United Kingdom as the foundation upon which our industry depends.

D. P. CLOUGH (Roche Products Limited); W. DAWSON (Lilly Research Centre Ltd); JOHN HUGHES (Parke-Davis Research Unit); TREVOR JONES (Wellcome Research Laboratories); P. S. RINGROSE (Pfizer Ltd); A. W. SIM (Organon Laboratories Ltd); M. J. TURNBULL (ICI Pharmaceuticals); A. M. WHITE (Ciba Geigy Pharmaceuticals)

Association of the

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12 Whitehall,
London SW1A 2DY, UK*

... and at NIH

SIR—One can only hope that your News report on the proposed new National Institutes of Health policies ("NIH propose cost benefit rules in grant reforms" by Christopher Anderson 348, 469; 1990) will ring alarm bells in the US scientific community and that a responsible scientific organization, such as the National Academy of Sciences, will be called in to create some order.

The NIH grant system, which for decades functioned reasonably well, is near chaos. Why? In part because the federal research budget has not grown at the same rate as the number of meritorious applications and their cost, but also because it became subject to conflicting, politically motivated policies, which have ignored the basic rules of arithmetic. With the new policy proposal, there is promise of still more policies that conflict with the rules of arithmetic — and the outlook is total chaos.

The solid numbers with which the extramural grant administration has to work are the annual federal appropriation and its fractional allocation to each programme. Earmarking for specific purposes — such as Alzheimer's disease and AIDS — which rarely involves additional funds, decreases, of course, the fractional allocation to other programmes. If, in addition, one decrees that the NIH should award 5,000 new grants a year, one must also decide on time and dollar limits of individual grants that can be accommodated within the annual allocation. The effects of neglecting to do so are now obvious: it seems to have been forgotten that 5,000

three-year grants mean a 15,000 grant load per year, but 5,000 five-year grants mean a 25,000 grant load per year. Since the decision to set a target for the number of new grants was made at the top, whereas the decisions on the duration and the dollar amount of each grant are basically made at the grass-roots level of the study sections, we faced a situation in which as the number of new grants and their cost grew larger, the grant periods also became longer — and after a few years finding funds for new awards became nearly impossible. Contrary to some of NIH's public statements, individual NIH programmes do not now fund 20–25 per cent of the approved applications. We have seen the applications rated in the upper 10 per cent go unfunded.

Now the new guidelines propose two significant measures: (1) to eliminate downward negotiations and (2) to consider the total cost of the project, including overhead, in deciding on an award. The first, taken together with the policy of maintaining 5,000 new awards a year, is a prescription for disaster. Downward negotiations did a great deal of damage to individual programmes, but they at least were a safety valve which allowed both established programmes to limp along and some new to get started. If they are eliminated, we have the following equation to reckon with: 5,000 new four-year grants a year mean an annual load of 20,000 grants; therefore the average allowed cost per grant has to equal the annual appropriation ÷ 20,000. Without a mathematically precise central control of individual grant costs, the two proposed policies are mutually exclusive. Yet a central control

of the duration and dollar amount of each grant would be a significant departure from past practices and would almost certainly involve downward negotiations.

The uncontrolled system where the number of new grants, their size and duration were at the discretion of each programme worked reasonably well. If control is indeed necessary, it should at least be sensible and transparent — and that means complete, so the programmes don't have to play one rule against another and against the unpredictable recommendations of the peer review groups. A simple formula assigning to each programme x grants for y years at $N \pm m$ dollars each would suffice to restore order.

The second — cost benefit — rule may have the effect of developing such a formula, but in a way that at the same time undercuts the peer-review system. How will one measure the cost-effectiveness of one research project against another? Is future NIH research to be done by the lowest bidder? If one departs from a single merit scale, with all its faults, and bases decisions on grant funding on a bivariate distribution of merit and cost-effectiveness ranking (if not trivariate, if one considers earmarking), how does one prevent it from becoming a game of Russian roulette? The implications of the proposed reforms are enormous. They bear all the earmarks of not having been thought through. They need to be discussed.

OLEG JARDEZKY

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The Smithsonian's tropical niche

Alun Anderson

The Smithsonian Tropical Research Institute in Panama is the best equipped and most productive tropical research institute in the world. A visit to the institute reveals some of the ingredients of its success.

THE closest a visitor to the Smithsonian Tropical Research Institute (STRI) in Panama comes to the popular vision of research in the tropics is on the verandah of an old wooden building above the landing stage on Barro Colorado Island. Behind are 1.5 square kilometres of dense tropical forest, covering an island that has been a nature reserve under continuous study by US scientists for over 70 years. Below is a vast man-made lake forming the central part of the Panama Canal.

But this building is now a part of the past; it has been replaced as the island's meeting place by a vast room without a view, a concrete air-conditioned cafeteria which can seat well over one hundred people under its neon strip lights. Close by are modern concrete residences with bright blue roofs. Unromantic though they may be, the new buildings sum up the past decade of STRI's achievement. Like no other place in the tropics, STRI has succeeded in making tropical research possible for beginners; providing everything from molecular biology laboratories to computer databases within a couple of hours of an international airport. In a busy year more than 300 researchers will come here, outnumbering STRI research staff ten to one.

More than 1,500 papers and theses have now been published on the subject of Barro Colorado Island alone. But still only the surface has been scratched and to the visitor's many naive questions a frequent answer is "No one has really looked at that yet". Examples are everywhere. Take the leafcutter ants — endless columns of them criss-cross every path on Barro Colorado Island, each ant carrying a quarter circle of cut leaf, held aloft like a little green flag. Leafcutter ants probably consume more foliage than all of the island's vertebrates put together, yet little is known of their impact.

But the biggest surprise about STRI is also the most immediate. The drive in from the airport reveals many of the familiar sights of Latin America: brightly painted buses which are crowded past capacity, young unemployed men who have no better way to make a dime than to try and wash your car windscreen in the seconds that you stop at a red light, and tumbledown shanties just down the road from magnificent apartment blocks. Then you arrive at STRI's headquarters, set around a huge tree on a little hill, just inside the border of the old Canal Zone.

Here, with the new laboratories and conference centre provided by the Tupper family (of 'Tupperware' fame), is a little piece of the United States — a US-funded institute, dominated by English-speaking foreigners, right in the middle of a Latin developing nation. For 70 years, surviving dictatorships and a US military invasion, biologists have done their work here. Surviving has not always been easy.

Outliving dictators

STRI's most anxious hours began on 20 December 1989, when at one in the morning, the first shells fell from US howitzers on Panamanian military headquarters. When the shooting finished, 22 years of tyranny were at an end and General Manuel Noriega was on his way to Florida to stand trial for drug trafficking — a curious end for a man whose first job was as a laboratory technician.

This was not the first crisis for Ira Rubinoff, STRI's director since 1974, and his assistant director for external affairs, Elena Lombardo. They carried STRI through the embarrassment of the US economic boycott when STRI could not pay rent or taxes; and through the collapse of the banking system when they could not pay their workers. Rubinoff also saw STRI through its other most difficult hour — the renegotiation of the canal treaties.

STRI owes its existence to the canal. After the French attempt to link Atlantic and Pacific ended in misery, with thousands dead from malaria and yellow fever the United States moved in, backing Panama's secession from Colombia in exchange for "absolute sovereignty" over a ten-mile wide Canal Zone that would cut Panama in two. By 1914 the canal was completed and a nature reserve, later to be handed over to the Smithsonian, had

been established within the US zone at Barro Colorado Island.

That arrangement could very easily have ended in 1977 when the Carter administration renegotiated the treaty, promising that the return of the canal would be complete by the end of the century. But Rubinoff, with help from the US State Department, "dusted off an old Hemispheric Convention on wildlife preservation that Panama and the United States had once signed" and succeeded in making Barro Colorado Island a reserve and STRI its steward. Later, in 1985, Rubinoff persuaded the Panamanian government to recognize STRI as an 'international mission' virtually ensuring its presence far into the future.

Panamanian relations

STRI's relationship with Panama now runs smoothly. There is none of the xenophobia of Brazil, where foreign researchers may have to find an equal number of Brazilian counterparts before beginning a project. STRI does help local researchers, providing some 50 scholarships a year, from predoctoral to postdoctoral level, half of them taken by people from Latin America. Numerous conservation and educational efforts within Panama are also supported, but cautiously. The stress has been on expert help for "efforts already going on" rather than on initiating new projects that might be seen as an interference. STRI's greatest gift to Panama is certainly its library, its 20,000 volumes, 1,000 journal subscriptions and electronic link to databases in Washington making it the best biology library in Latin America. Astonishingly, it attracts over a thousand visitors a month, some coming from other Latin American countries simply to use its facilities.



STRI's tiny island in a shallow, coral sea. The island is little more than a heap of sand and coral rubble created, so the story goes, by a Kuna Indian from the next island, scarcely a hundred yards away, because he wanted to escape from his in-laws. STRI's presence here is dependent on maintaining good relations with the Kuna, a group of some 45,000 native people who live in a chain of several hundred low islands along the Caribbean coast. Thanks to the Kuna, who live on the islands to avoid mosquitos and sandflies and practice only slash-and-burn agriculture in their tribal lands along the mainland, a large area of coastal rain forests remains intact. Photo by C. Hansen, STRI.

But there are still almost no Panamanians among STRI's senior scientific staff. One Panamanian explains that there is a sad old joke that the standard ambition for a talented University of Panama student is "Emigrate!". With university salaries around \$400 a month for a professor, a scientific career in Panama does not appeal. But that may be changing, given the number of young Panamanian students working as assistants on STRI projects. There is little pressure from Panama for STRI to do more. Rather, where there is criticism of STRI's efforts to integrate itself into Panama, it comes from US researchers.

Some young researchers are plainly embarrassed that at meal times, tables split into those where English is spoken and those where Spanish is spoken. Some also admire the vigorous example being set across the border in Costa Rica. There a consortium of US universities, the Organization for Tropical Studies (OTS), maintains facilities designed to introduce students to the tropics. Dan Janzen, one controversial researcher associated with OTS, has not only helped persuade the government to invest in national parks, but has set up a National Biodiversity Institute which trains Costa Rican 'para-taxonomists' who will produce an inventory of their own country's flora and fauna. Strangely, there is almost no collaboration between STRI and OTS, although researchers claim change is imminent.

A narrow isthmus

The principal interest of researchers at STRI is evolutionary biology and ecology and much of the work is small scale. But pressure from the disciplines that STRI represents and growing concern about the impact of man on the environment is bringing about a shift towards bigger science with a focus on global change, greenhouse gases, and the understanding of biogeochemical cycles. In particular, the need for information about past large-scale changes has gained new importance with STRI adding to its staff palaeobotanists who can help reconstruct past climates and flora.

As every pamphlet about STRI points out, the institute offers a unique advantage by sitting on a land bridge which, three million years ago, separated the Atlantic land masses. That creates many opportunities to study evolution in action, which STRI researchers have been quick to exploit. Among them, for example, is Haris Lesios who has compared genetic changes in Pacific and Atlantic populations of sea urchins to test the accuracy of the 'molecular clock'.

But STRI's locations also means that the nearby forest is new, in evolutionary terms, and may not be representative of the much older and more diverse tropical

forests, like those in south-east Asia.

STRI is now looking out for collaborations elsewhere in the tropical world that will help prove if the extensive work performed at STRI can be generalized to less well-known areas. With Princeton University, STRI is set to gain a 50,000-acre site in Kenya that has been left by a Princeton donor. Another site in Ecuador looks likely to become available. The first major international project, a new Center for Tropical Forest Science, has already taken off under the guidance of Stephen Hubbell, of Princeton University.

At its heart is the largest project on tropical community ecology ever attempted, a 50-hectare (half-square kilometre) plot of land on Barro Colorado Island where a quarter of a million trees and shrubs were labelled and mapped a decade ago. Since then the plot has been censused twice more — in 1985 and 1990 — and growth of each of 303 species correlated with such variables as soil, moisture, light and proximity to other trees.

Matching plots were added in 1986 in Malaysia (the plot turned out to contain over 800 species) and in 1988 in southern India. With the new centre, Hubbell hopes to get other collaborative projects going elsewhere, all sharing the same measurement methods and standards. The aims are twofold; to understand the dynamics of tropical forests and to come up, very rapidly, with models for the sustainable management of tropical forests. Large plots, Hubbell thinks, will provide quick knowledge of the best growing conditions for many different kinds of tropical tree, far more than those presently under intensive study in plantations.

Change natural and unnatural

STRI researchers have been studying natural change for years, and finding much to study despite the old conception of the tropical world as a place without change or seasons. That has not, however, given them great confidence to predict what will happen to tropical ecosystems as man-made climatic changes make their appearance. Field experiments tailored to this purpose, such as artificially changing the levels of carbon dioxide available to plants in a natural habitat, are only just beginning.

Hindrik Wolda was one of the first to show that tropical populations are ever changing. He has spent the past 17 years monitoring the day-to-day variation in the many species of insects drawn to light traps he set up on Barro Colorado Island. The result is, he says, that "insect populations fluctuate here just as much as they do anywhere". But it is more difficult to find out why, given that so little is known about the life histories of the insects he captures. Most, he says, are simply "endemic to my light trap".

The most dramatic changes seen by

STRI researchers resulted from the severe El Niño event of 1982–83. In the forests, less rain fell and 15 per cent of the trees in Hubbell's 50-hectare plot died. In the reefs, higher water temperatures caused coral to bleach — expelling the photosynthetic algae that live inside their tissues. Recently, corals have been seen to bleach again throughout the Caribbean, and perhaps elsewhere. Whether this is an early sign of rising sea temperatures produced by global warming or a natural variation is disputed; underwater observations are only as old as inexpensive scuba gear.

In the same year as the El Niño, STRI scientists also charted another alarming change as the long-spined sea urchin *Diadema antillarum* began to die throughout the Caribbean. No one knows what is to blame but the result has been the almost total annihilation of what was a common inhabitant of the reef. Without the grazing sea urchins, algae are growing over reefs, preventing larvae from settling. In areas such as Jamaica, where fishermen catch too many of the herbivorous fishes that might otherwise compensate for the loss of the sea urchin, the end may now be in sight for shallow-water reefs.

Far more long-term records are clearly needed to understand the forces behind population booms and crashes. Thanks to STRI's long residence in Panama, some records already exist. On Barro Colorado Island a long-term Environmental Sciences Program measures, rain, wind, temperature, the time of fruiting and flowering and other key environmental factors. Such projects are not easy to run in an institute dominated by independent investigators but STRI is looking for ways to ensure that such projects can outlast one individual's tenure (or enthusiasm).

Long-term information on the extent of past changes is being mined from coral cores, archaeological studies and from palaeobotanists joining STRI. First among them is Dolores Piperno whose work on phytoliths — microscopic grains of silica formed in plant tissue that are characteristic of a particular species — charts the rise and fall of particular plants. Environmentalists will probably not be heartened to hear that humans have already destroyed the Panamanian forests once. Phytoliths obtained from lake cores show that human agricultural activity began 11,000 BP (with the appearance of characteristic weed species) and much of the forest was gone by 4,000 BP. The forest that now covers the Darien peninsula owes its existence to yet further deprivations by humans. After the Spanish conquest, disease and slavery so disrupted the agricultural system that these areas now appear as if they were never touched by human hand. □

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Making sense of binary metal alloys

A Cray-led attack on the structure of the binary alloy of nickel and platinum seems to be most easily interpreted by reference to empirical rules put forward by Hume-Rothery more than half a century ago.

WILLIAM Hume-Rothery, the metallurgist who in the 1930s did much to develop rules for the structure of metallic alloys based on what was then being learned of electronic structure, had the misfortune to be stone-deaf. Among other things, his affliction meant that, over several decades, he was not invited to be a member of an Oxford Senior Common Room until the late A. S. Russell, once active in early radioactivity (with Fajans and Soddy, for example) persuaded the members of the college called Christ Church — it is also a cathedral — that they could stomach the occasional social embarrassment of a deaf man at dinner.

By then, 1944 or thereabouts, Hume-Rothery had become skilled at using his rasping monotone to get his points across; many cohorts of students were persuaded that what matters in the determination of the structure of an alloy, its electrical conductivity and even its inclination towards order or disorder, are differences of atomic (not ionic) size, electronegativity and the electron-per-atom ratio. One of his rules, for example, is that atoms differing in size by more than 15 per cent will not form solid solutions, which rather begs the question of what is meant by "size" when atoms may be embedded as ions in a sea of moveable electrons. Nevertheless, Hume-Rothery claimed no more for the rules than that they were empirical generalizations cast in a language ripe for explanation. Yet the explanations have been slow to come.

There is, for example, the question of when an equimolar binary alloy is likely to form crystals in which neighbouring lattice sites are alternately occupied by different species of atoms. The question is obviously of practical importance, given that a material such as ordered β -brass is evidently very different from its components, copper and zinc. Conventional wisdom (acquired in the past decade, after Hume-Rothery's time) is that ordering in transition-metal alloys is probable when the d conduction band is roughly half filled by electrons, and is improbable when the Fermi energy (that of the most energetic electron) is near either the top or the bottom.

Now F. J. Pinski and five other theoreticians have dealt in some detail with what appears to be an exception to that rule — the equimolar alloy of nickel and platinum, $\text{Ni}_{0.5}\text{Pt}_{0.5}$, which has a d -band system almost filled with electrons, but

whose structure has a high degree of short-range order. Part of the interest of their article is that it attempts, apparently successfully, to calculate the influence of Hume-Rothery's three factors.

For what it is worth, alloys of Ni and Pt are ferromagnetic at sufficiently low concentration of Pt, which has made the system a test-bed for studies of the interaction between chemical and magnetic ordering. (These days, no doubt, the possibility that a similar interplay is involved in the influence of doping cations in high-temperature superconductors should not be overlooked.) But the paper of Pinski *et al.* (*Phys. Rev. Lett.* **66**, 766; 1991) is also remarkable in that its six authors give six different affiliations (the universities of Cincinnati, Messina, Warwick and Bristol, the Sandia National Laboratory at Livermore and the Oak Ridge National Laboratory in Tennessee).

Pinski *et al.* choose to tackle the problem from the difficult end of the spectrum, by treating first the case of a randomly disordered solid solution of Ni and Pt, and estimating its instability with respect to microscopic transformations that lead to short-range or local order. But even if they had set out in the other direction to estimate the instability of the ordered phase with respect to disordering processes (entailing assumptions, perhaps invalid, about the structure of the ordered phase), they would have had to devise some way of representing self-consistently the electrical potential to which the d -electrons are subjected on account of the chance that a lattice-site occupied by Ni in the ordered state may, in reality, be occupied by Pt.

The trick, called the coherent potential approximation, is a kind of mean-field theory which, like all mean-field theories, is characterized by the level in the argument at which the averaging is carried out. Plainly it would make no sense to suppose that the electrical potential emanating from each lattice site is the average of the potentials of the cores of Ni and Pt, for that would exclude the possibility of short-range ordering. Moreover, because the likelihood of short-range ordering is critically a function of the temperature, it is as well to include the lattice vibrations from the start. The result is a way of handling the electronic potential both as a determinant of the degree of short-range order and as a consequence thereof.

Interestingly, while thanking both the Ohio Supercomputer Center and Cray Research Inc., Pinski *et al.* present their results as a two-dimensional plot of the short-range order parameter for various directions in reciprocal-lattice space — and find themselves following Hume-Rothery in the manner in which they say what it means. But one conclusion is that the predominant effect in the ordering process is that of nearest neighbours (and that the energetic consequences of next-nearest neighbours are only 10 per cent as great).

Another striking feature of the calculation is that Pt atoms contribute to the random alloy electron states chiefly in the lower half of the d -electron band, with Ni atoms supplying the remainder. The argument then goes that, in the real world, states from the two sources will hybridize with each other, forming energetically favourable chemically bonding states. This, Pinski and his colleagues say, is the source of the ordering propensity in NiPt. And as the states that hybridize, or mix, in the ordered structure are mostly both below the Fermi energy (which measures the extent to which the band is filled), the exact location of the Fermi level is not particularly relevant to the ordering propensity. That, the authors say, explains why the half-full band criterion — last decade's received wisdom — is irrelevant, in this exceptional case, to the ordering propensity of NiPt. In Hume-Rothery language, this is a way of saying that the electron/atom ratio is immaterial.

Pinski *et al.* go one step further towards Hume-Rothery's position in taking up the question of the difference of size between the Ni and Pt atoms in the ordered alloy. In the quantum picture of a lattice structure held together by electrons, the 'size' of the entities occupying neighbouring lattice sites has no meaning on its own. What the calculation (and the real world) provide is the lattice spacing, which is a function of the crystal structure as a whole, in which the degree of short-range ordering is merely another parameter. But in the randomly ordered NiPt alloy, Ni atoms occupy more space, and the larger Pt atoms less space, than in the respective pure metals. So short-range ordering, where one kind of atom tends to be surrounded by atoms of the other kind, here tends to relieve strain. Thus in principle, if not in detail, is Hume-Rothery vindicated again.

John Maddox

A system for synapse control

Regis B. Kelly

To reductionists, understanding the brain means knowing the molecules involved in forming synapses during development and in modifying synapses as a function of use. A considerable step towards this goal has been achieved by Han *et al.*, whose report¹ appears on page 697 of this issue. By transfection, Han *et al.* increased the level of a membrane-associated synaptic vesicle protein, synapsin IIb, in a neuronal cell line. Before transfection the cell line puts out neurites that have nerve terminal-like swellings or varicosities, but the varicosities contain few synaptic vesicles and they do not form synapses. After transfection there is a dramatic increase in the number of synaptic vesicles per varicosity and in varicosities per neuritic extension; moreover — and most unexpectedly — the cells can now form morphologically identifiable synapses with each other (see figure). The level of synapsin IIb therefore seems to control the ability to make synapses in these cells.

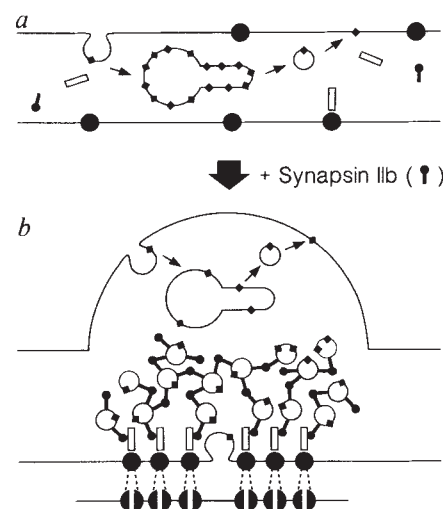
Nerve cells communicate both with adjacent nerve cells and with those some distance away. Long-range communication, analogous to endocrine secretion, is due to the release of the contents of large vesicles that have electron-dense cores in electron micrographs². These vesicles are morphologically indistinguishable from the secretory granules of endocrine cells. The site at which they fuse to the plasma membrane is not tightly regulated, and their contents interact with any other nerve cell within diffusion range that has the appropriate receptors. Short-range communication with adjacent cells is very different and involves specialized cell-cell contacts, the synapses. In the synapse, the signalling molecules are packaged in small electron-lucent synaptic vesicles, the fusion site of the vesicles being restricted to a tiny region of plasma membrane called the active zone; the synaptic vesicles are held in close vicinity to the active zone and the active zone is physically linked to the receptor-rich, post-synaptic region of a target cell by extracellular bridges. Discovering the proteins that connect the synaptic vesicles to active zones and active zones to postsynaptic receptors is crucial to understanding synaptogenesis and probably also synaptic plasticity.

Synapsins

Because synaptic vesicles are connected to active zones but dense-core vesicles apparently are not, one expects to find a class of nerve terminal-specific cytoskeletal proteins that binds to synaptic vesicles but not to dense-core vesicles. The synapsins, peripheral membrane

proteins of synaptic vesicles, seem to have the expected properties³. There are four of them, all very similar in amino-acid sequence. Synapsins Ia and Ib, which make up 0.4 per cent of total brain protein, are generated by alternative splicing of one gene; synapsins IIa and IIb (0.2 per cent of total brain protein), by splicing of another.

Largely through the efforts of Paul Greengard and his colleagues, the distribution and properties of the synapsins have been found to be consistent with involvement in tethering synaptic vesicles. Synapsins appear in development during synaptogenesis, are targeted to nerve terminals and localize to the regions of the nerve terminals at which synaptic vesicles reside. They are not associated with dense-core vesicles which, although present in nerve terminals, do not release their contents exclusively at the active



A model of synapse formation based on the work of Han *et al.*¹ *a*, In a normal cell, most of the synaptic vesicle proteins (diamonds) are in a precursor organelle, probably an endosome represented here as a spherical vesicle with a tubular extension. Membrane proteins recycle from the cell surface to endosomes by way of small transport vesicles. Adhesion molecules on the cell surface (large solid circles) diffuse freely in the plane of the membrane. *b*, After transfection to increase the levels of synapsin IIb, endosomes are depleted of their synaptic vesicle proteins, synaptic vesicles of uniform diameter are formed and linked to a small region of the cell surface in which adhesion molecules are now clustered. The adhesion molecules are linked to the aggregate by filamentous proteins (parallel lines). The clusters of adhesion molecules are themselves linked (dashed lines) to postsynaptic receptors (split solid circles) to maintain the neurotransmitter release site in apposition to the postsynaptic elements. The linking molecules appear fuzzy between pre- and post-synaptic membranes in the electron micrographs of Han *et al.*¹. One possibility is that the active zone is generated without new protein synthesis by the assembly of pre-existing elements into a synapsin-rich macromolecular matrix. The matrix holds the vesicles in the vicinity of the active zone, and holds the active zone in apposition to the postsynaptic receptors.

zone². The synapsins also have the physical characteristics of proteins associated with the cytoskeleton. They are lollipop-shaped, with a head (15–14 nm) and a filamentous tail (35–47 nm), readily form aggregates, and interact with filamentous actin. The filamentous protein that is seen to link synaptic vesicles and actin in quick-freeze electron micrographs of nerve terminals is thought to be synapsin^{4,6}.

The synapsins have been implicated in the functioning of nerve terminals as well as development because synaptic transmission is blocked by the dephosphorylated form of synapsin I (ref. 7). Phosphorylation of synapsins occurs during synaptic transmission and inhibits the blocking ability of synapsin I.

Transformation

If synapsins do indeed play a part in connecting synaptic vesicles to the active zone, then raising their expression level might increase the number of vesicles in nerve terminals. Han *et al.*¹ verify this prediction by transfecting DNA encoding one of the four synapsins, synapsin IIb, into a neuronal cell line, NG108-15, deficient in this synapsin. This cell line, a neuroblastoma-glioma hybrid, was first described over 15 years ago but has not been extensively exploited despite its remarkable properties. When grown under differentiating conditions, NG108-15 cells extend long processes that have synapse-like varicosities packed with dense-core vesicles but few if any synaptic vesicles. They do not form morphologically identifiable synapses with each other when grown alone, but accumulate synaptic vesicles and form physiologically functional synaptic contacts on muscle cells when co-cultured with them. When NG108-15 cells are transfected with DNA encoding synapsin IIb, a remarkable transformation takes place. The number of synaptic vesicles per varicosity increases 12-fold, the number of varicosities per neurite increases threefold, and the varicosities make specialized contacts with each other that morphologically resemble synapses. Surprisingly, the introduction of a single protein into the cell seems to cause extensive architectural rearrangement leading to synapse formation.

Increasing the concentration of synapsin IIb 24 times that of controls causes a greater than 30-fold increase in the numbers of synaptic vesicles in varicosities. Because the level of a synaptic vesicle protein, synaptophysin, only doubles, the accumulation of synaptic vesicles must be due to a redistribution of components of the vesicles. Such an accumulation is easy to explain if synapsin IIb anchors to the active zone and then traps synaptic vesicles. But where do the synaptic vesicles come from? They could be distributed uniformly throughout the

cytoplasm of the NG108-15 cells before transfection and so be missed in electron micrographs. Alternatively, if they are indeed generated from early endosomes, as recent data seem to suggest⁸, increased expression of synapsin IIb might favour their formation from an endosomal precursor (see figure). Whichever is true, an unavoidable conclusion is that accumulation of synaptic vesicles is not attributable to net synthesis of their component proteins.

What is less easy to explain is how transfecting a cell with DNA for a single protein can also induce the accumulation of dense-core vesicles in varicosities, the generation of varicosities and the formation of the cell-cell connections resembling synapses. But a single protein can cause considerable changes in cell architecture when raising its concentration triggers the assembly of a macromolecular network. For example, when the cytoskeleton of red blood cells self-assembles into a stable network, the membrane anchor may be limiting⁹. The expression of a single cytoskeletal protein, villin, triggers the appearance of microvilli¹⁰. And the calcium-dependent adhesion molecule E-cadherin (also called uvomorulin or L-CAM) is essential for the development of polarity in epithelial cells, inducing the re-distribution of cytoskeleton and the membrane-associated sodium pump when introduced by transfection into fibroblasts¹¹. A key feature of these and other such rearrangements is that stable macromolecular networks assemble out of pre-existing components. Increase in stability of the proteins raises their level in each cell without changes in the rate of synthesis.

If these examples are valid analogies to what is happening in transfected NG108-15 cells, then higher levels of synapsin IIb might be inducing varicosities and synapses by triggering the assembly of active zones from pre-existing elements (see figure). One finding by Han *et al.*¹ favours this idea. Elevating the level of synapsin IIb by transfection also raises the level of synapsins I and IIa fourfold. Such a result would be predicted if transfection induces a stable macromolecular network that includes among its constituents the

other two synapsins. Such a mechanism would of course be much more attractive if we knew that the increases in levels of synapsin I and IIa were due to stabilization rather than new synthesis.

Both accumulation of synaptic vesicles at varicosities and the increases in endogenous synapsins are consistent with a model in which synaptogenesis is triggered by self-assembly from pre-existing components. The advantage to the neuron of assembling a synapse in this way is that synaptogenesis can be triggered locally, liberating synaptic terminals from depen-

dence on new protein synthesis in a cell body that can be quite distant. Local control of synaptic morphology also allows changes to take place in minutes rather than hours and days. The beauty of the work described by Han *et al.*¹ is that it encourages conjectures about synapse formation while at the same time offering an experimental system in which to test them. □

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ACTIVE GALACTIC NUCLEI

Light hidden behind a bushel

Paul Hewett

BL LAC objects represent one of the most extreme types of active galactic nuclei (AGNs) — a family that also contains the more familiar quasars and some radio galaxies. BL Lacs are characterized by large-amplitude, rapid luminosity variations and highly polarized continuum emission from radio to X-ray frequencies; they also lack the strong optical and ultraviolet emission lines usually taken as the signature of AGNs. Ostriker and Vietri¹ proposed an ingenious model in which the strong continuum emission and the absence of strong emission lines result from gravitational lensing by stars (microlensing) in galaxies along our line of sight to the BL Lacs. More recently they cited² the existence of two BL Lacs seen projected within the optical extents of relatively nearby elliptical galaxies as further evidence to support their model. But on page 676 of this issue³ Gear questions the feasibility of this concept.

Gear reviews recent observational results relevant to the structure of BL Lacs and concludes that the microlensing model is probably not viable because the continuum-emitting regions are too large to be lensed effectively. Ironically, in a paper to appear in next week's *Nature*⁴, Gopal-Krishna and Subramanian present theoretical calculations which suggest that microlensing explains not only the spectral characteristics of BL Lacs, but also their rapid variability, thought by Ostriker and Vietri to be a property of the source object.

Gravitational lensing by whole galaxies and clusters of galaxies is now well established as a powerful tool for the investigation of distant mass concentrations, and also as offering a remarkable method for seeing intrinsically faint galaxies at very high redshifts⁵ (equivalent to great distances). Perhaps the most impressive application has been the identification of the spectacular 'arcs' — the highly distorted images of high-redshift ($z \approx 1$) galaxies seen through the cores of rich galaxy

clusters of intermediate redshifts, $z \approx 0.3$. The spectra of the lensed galaxies are allowing the degree of star-formation in normal galaxies at redshifts $z \approx 1$ to be investigated for the first time. The extension of this work by Tyson and others to the identification of many more 'arcs', the slightly distorted images of background galaxies also due to lensing by the same rich galaxy clusters, may allow a direct mapping of the dark matter distribution within rich clusters.

The key factor in determining whether microlensing by individual stars in foreground galaxies can radically alter the appearance of distant AGNs to produce BL Lacs is the size of the continuum-radiation-emitting region. It is well established that the emission-line-generating regions of AGNs are far too large (at about 1 parsec or 300 million light years) to be significantly influenced by stellar-mass lenses. The motivation behind Ostriker and Vietri's microlensing model, however, was that the large-amplitude, very-rapid variability observed in BL Lacs implied that their continuum-radiation-producing regions are less than 10^{-3} pc. In that case, lensing by stellar-mass objects could be significant. So microlensing of a distant AGN would give the strong continuum emission and weak line emission characteristic of a BL Lac object.

The appearance now of two apparently contradictory papers^{3,4} reflects our lack of quantitative knowledge of the structure of BL Lacs and AGNs in general. Gear's paper reviews a number of recent investigations and points out that structures on scales from 10^{-3} pc to several parsecs are important. Radio observations with high dynamic range and that extend to high-resolution are necessary to reveal how features on different scales in the jets of AGNs are related and to determine the evolution of shocks as they propagate along the jets. Gear shows that in a simple jet model, the width of the shocked region, one candidate site for continuum

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radiation generation, is too large to be significantly affected by microlensing once it is several parsecs from the B1 Lac core. But observations⁶ of the radio-jet in the Virgo cluster galaxy M87 suggest that jet structure may be more complicated. Many filaments are evident in the M87 jet, stimulating some theoreticians to consider models in which jets contain helical filaments and the propagating shock results in emission from regions much smaller than the jet width⁷, a picture which would provide additional motivation for Gopal-Krishna and Subramanian's study. Progress in our understanding of jets can be expected as the upgraded British MERLIN radio interferometer and the Australia Telescope begin to produce high-dynamic-range maps with sub-arcsecond resolution of the nearest radio-jets.

Despite the spectacular examples of the lensing phenomenon, it has proved difficult to find unambiguous statistical evidence for the influence of foreground mass concentrations, including galaxies, on our view of extragalactic background populations such as BL Lacs. In part this is because of the small number of BL Lacs and the substantial effort required simply to measure their redshifts, but more fundamentally, gravitational lensing produces a change in the observed properties of the objects. In the simplest case this may be an apparent increase in brightness for the fraction of the underlying population significantly affected by lensing. For the case of an AGN projected close to the image of an intervening galaxy, to be expected under the hypothesis that galaxies are lensing AGNs, the AGN may no longer appear to be an unresolved point of light, a criterion often used to determine whether an object is an AGN.

The probability of including objects affected by lensing in surveys can thus be altered in significant and often subtle ways, making the interpretation of anomalous numbers of AGN-galaxy projections difficult. Forearmed with this knowledge, and taking advantage of the availability of digital data, several groups are now undertaking surveys with broadly based and completely specified selection procedures. The application of the results to questions such as whether there really is an excess of BL Lacs seen close to foreground galaxies will help establish how important gravitational lensing is in affecting our view of the Universe. □

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John Bardeen (1908–1991)

THE unique honour of two Nobel prizes in physics understates, if anything, the impact which John Bardeen, who died on 30 January, had on modern science and technology.

Bardeen's undergraduate and master's degrees were in electrical engineering at Wisconsin, and he spent three years as a geophysicist before going to Princeton for a PhD with Eugene Wigner (1936), and thence to Harvard as a Junior Fellow. Thus he managed to work in his formative period with both of the founders of American solid-state theory, Wigner and John van Vleck. In these years he carried the band theory of the alkali metals, begun by Wigner and Seitz, to the level of practical calculations of equations of state, resistivity and surface barriers. It was characteristic of him from then on to refer drily to the most recondite of theoretical ideas as "calculations".

After the war, which he spent at the Naval Ordnance Laboratory, he was brought to the Bell Laboratories to work in the group that J. B. Fisk and W. Shockley hoped would produce the active semiconductor device which, indeed, W. Brattain and Bardeen discovered, in the dying days of 1947, and named the transistor. This period of work on semiconductor physics also produced several important theoretical developments such as a vital summary paper with G. Pearson introducing, *inter alia*, the impurity band, and the concept of deformation potentials, published with Shockley. Oddly enough, the specific theoretical ideas on surface states which were part of his contribution to the transistor may have been only a rough "demonstration of possibility", yet there is no doubt that this contribution was vital.

Bardeen had made the first of his three theoretical attempts at explaining superconductivity in 1944; the second came in 1950 when he learned about Serin and colleagues' results on the effect of isotopic mass on the transition temperature in mercury. This and Fröhlich's similar theory had become discredited by 1953; yet Bardeen continued, convinced that the solution was close and that the key lay in careful study both of previous theoretical insights and of the experimental picture. Many great theorists, from Einstein to Feynman to Landau, had tried to understand superconductivity, but few or none had remained as close as Bardeen to the developing experimental situation.

The need for freedom from "transistoritis", as he called it, led to his leaving Bell Laboratories in 1951 for the Univer-

sity of Illinois; I remember that even the 1956 Nobel prize festivities were seen by Bardeen as a not totally welcome interruption of the search for the theory of superconductivity. Finally, in early 1957, 46 years after Onnes's discovery of the phenomenon, and in company with Bob Schrieffer, his student, and Leon Cooper, a postdoctoral associate, he created the successful BCS theory. For at least a decade, a sequence of follow-up developments poured out of Bardeen and his students and associates; a second Nobel prize came in 1972.

Yet a third or fourth career came when he associated himself with the infant Xerox corporation as a consulting director in 1960, and helped oversee its transition from start-up company to corporate laboratory. Even after retirement Bardeen retained strong scientific interests. A characteristically opaque and controversial theory of sliding charge density waves enlivened his later years, and may yet win out over more conventional ideas.

It is impossible to overstate the importance of the transistor and the semiconductor physics which flowed from it. The developments far overshadow both nuclear fusion and fission and have unquestionably had the largest economic and social impact of any idea in modern physics. Almost equally important is the BCS theory, which revolutionized nuclear and particle theory as well as solid-state physics: for instance, the broken symmetry concept behind the electroweak theory originated from BCS.

Bardeen's notorious parsimony with words frustrated many an interviewer and provided fuel for countless anecdotes. Nonetheless he was a warm and faithful friend, partnered by his universally beloved wife Jane. Bardeen enjoyed the courage of his convictions, represented for example by his resignation from the White House Science Council over President Reagan's 'star wars' speech, and his personal funding of the prestigious Fritz London prize for low-temperature physics. He leaves us two well-known physicist sons, William and James. His daughter, Betsy, now engaged in organizing information systems for the State of Massachusetts, is married to yet another physicist, Thomas Greytak.

John Bardeen was a happy, quiet, gentle giant — but a giant nonetheless.

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Beta amyloid resurrected

Alan F. Wright, Michel Goedert and Nicholas D. Hastie

THE report by Goate *et al.* on page 704 of this issue¹ reopens a debate over the genetic basis of one form of Alzheimer's disease. The authors find that a mutation within the gene for amyloid precursor protein segregates with the disease in two families. Although the results suggest that this genetic change could underlie the disorder, they are not yet conclusive.

Alzheimer's disease (AD) is the most common of age-related cerebral disorders. Its prevalence rises exponentially with age to reach at least one in five by the eighth decade², implying that genetic differences in the population play a minor role in its pathogenesis. But genetic factors can influence the age at which an individual manifests what may otherwise be an almost inevitable consequence of age-related decline. The heritability of the disease appears to increase as the age-of-onset falls³, the most striking example being a rare autosomal dominant form of familial AD. In 1987, St George-Hyslop *et al.*⁴ opened up the field by mapping the gene responsible for familial AD in four families to the long arm of chromosome 21. The most obvious 'candidate gene' for the disease, the amyloid precursor protein (APP) gene, mapped to the same broad region but was apparently excluded by the demonstration of several recombination events between familial AD and the APP gene in the same and other families^{5,6}. Goate *et al.* now suggest, however, that mutation within the APP gene could account for at least a proportion of early-onset cases.

The neuropathological hallmarks of AD are abundant amyloid plaques, cerebrovascular amyloid and neurofibrillary tangles. All of these features can be found to some extent in the 'normal' ageing brain. Also, most individuals with Down's syndrome reaching the age of 40 show them to the same extent as in Alzheimer's disease — the first indication of the importance of chromosome 21 in this context. Seven years ago, the partial sequence of the amyloid β protein⁷, the main protein constituent of amyloid plaques and cerebrovascular amyloid, was obtained. This led to the cloning of the APP gene which showed that the amyloid β protein is a 42–43 amino acid cleavage product of the larger precursor protein⁸.

Domains

The messenger RNA of APP can be spliced to produce three principal isoforms (APP695, APP751, APP770), the two larger of which contain a serine protease inhibitor domain. Several other domains are identifiable in APP, including a transmembrane region that supports

the idea of there being a membrane-bound protein capable of being cleaved physiologically into a large (> 100K), soluble, N-terminus fragment that contains the protease inhibitor domain in some isoforms, and a 9K membrane-bound, C-terminus fragment that includes most of the amyloid β protein region⁹. Secreted isoforms of APP containing the protease inhibitor domain are identical to the serine protease inhibitor nexin II¹⁰. One possible function — regulation of peptide growth factor-related proteases, which has implications for the maintenance of neuronal processes — is tentatively suggested by analogy with nexin II. The amyloid β protein seems to be an abnormal cleavage product of APP, because it contains the constitutive cleavage site and terminates within the transmembrane domain. A feature of the amyloid β protein structure is that it may contain β -pleated sheets, making it prone to formation of dense amyloidogenic aggregates that are resistant to proteolytic degradation and removal¹¹.

Goate *et al.* previously helped to confirm the localization of a familial AD gene on chromosome 21 by providing evidence of genetic linkage in six families¹². Further analysis of one of these families¹ showed that part of the region distal to the APP gene could be confidently excluded, and the APP proximal region previously thought to contain the gene for familial AD, could also be tentatively excluded. The region containing the APP gene was not excluded however, resurrecting the possibility of the APP gene as a candidate gene.

Goate *et al.*¹ then decided to sequence part of the APP gene that included the amyloid β protein sequence from an affected patient, perhaps spurred on by the recent identification of a mutation within this region associated with a rare Dutch type of hereditary cerebral amyloid angiopathy (HCHWA-D) (ref. 13). They found a conservative mis-sense mutation in exon 17, causing a valine-to-isoleucine substitution at a residue (position 717) within the transmembrane segment, only two residues beyond the C terminus of the amyloid β protein.

The mutation co-segregated with the disease within the family, which in itself is not surprising, but they did not find it in any of the 200 normal chromosomes analysed or in nine families with late-onset AD, arguing against the simplest explanation, namely that a conservative and functionally neutral substitution is present as a polymorphism in the population. However, 'silent' mutations that are individually rare, even family-specific, are

collectively not uncommon. This possibility became less likely when Goate *et al.* found the same mutation in two affected members of another family subject to early-onset familial AD, on a different genetic background. Analysis of eight other familial AD families revealed no further examples of the mutation. One fifth of the familial families surveyed carried this single mutational change, whereas less than one per cent of the general population carry the same APP allele¹.

The major questions that now have to be addressed are ones that are highly amenable to investigation by molecular genetic techniques. How common is the mutation at APP residue 717 in the general population and in early-onset sporadic and familial forms of AD? What other mutations, if any, are found in the APP gene? The ability to amplify exons from genomic DNA by the polymerase chain reaction and efficiently screen them for single base pair substitutions by a variety of techniques will result in swift answers to these questions.

Recombination

Why was the APP gene excluded as a candidate gene in apparently similar chromosome 21-linked familial AD families? It is clear that some familial AD families with early onset and autosomal dominant inheritance show no evidence of linkage to chromosome 21 markers¹⁴, neither do late-onset AD families^{14,15}. What is less clear is how the new results of Goate *et al.* can be reconciled with reports of at least seven recombination events between the APP and AD familial genes in families linked to chromosome 21 markers^{5,6}. The implication is that there must be another gene on chromosome 21, perhaps nearer to the centromere, that predisposes to familial AD. Genetic heterogeneity is almost the rule for diffuse clinical phenotypes and there must be several loci capable of influencing APP processing and turnover, if that is indeed a primary factor. It remains to be clarified why two loci with such large effects on susceptibility to disease should be located about 20 centimorgans apart on the same chromosome. Alternatively, as Goate *et al.* point out¹, reports of recombination between familial AD and the APP gene in chromosome 21-linked families may have been in error owing to factors such as non-paternity, mistyping, misdiagnosis or phenocopies.

What further evidence should be provided to establish or discount a primary aetiological role for APP in familial AD? There are strong parallels between this situation and the recent demonstration of a mutation in the prion protein gene which co-segregates with the disease in families with another early-onset but (unlike AD) transmissible form of dementia, Gerstmann-Sträussler syndrome¹⁶. The genetic findings were again suggestive but not

conclusive. Acceptance came with the dramatic demonstration that transgenic mice carrying the same point mutation as patients developed a spontaneous spongiform encephalopathy, similar to that found in the human disorder¹⁷. Will transgenic mice carrying the reported substitution at APP residue 717 develop amyloid plaques and neurofibrillary tangles? Similarly, will transgenic mice expressing the APP mutation found in hereditary cerebral haemorrhage with amyloidosis (HCHWA-D) develop cerebrovascular amyloid deposits? It is striking that the APP mutation occurring in HCHWA-D is associated with large numbers of β -amyloid deposits in cerebral blood vessels and only small numbers in the neuropil, with no neurofibrillary tangles and a different clinical picture from AD. Further information on the effects of such mutations on APP processing could be obtained using cultured cell systems¹⁸.

Precedents for the occurrence of serious neurological consequences arising from mutations in amyloidogenic proteins are growing steadily and now include familial amyloidotic polyneuropathy¹⁸, two different types of hereditary cerebral haemorrhage with amyloidosis (HCHWA-I, HCHWA-D) (refs 13 and 19), Gerstmann-Sträussler syndrome¹⁶ and some forms of Creutzfeldt-Jakob disease²⁰. There is the possibility that early-onset AD occurs in Down's syndrome simply because of over-expression of the APP gene by gene dosage. But argument by analogy is inadequate in cases such as this — nothing less than hard experimental evidence will establish whether or not the APP gene represents a gene for familial Alzheimer's disease. □

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Slow renewal of deep waters

Michael Grachev

LAKE Baikal, in east Siberia, the deepest and most ancient freshwater lake in the world, contains a fifth of the world's deposits of liquid fresh water. The lake and its remarkably pure water deserve thorough protection, which must be based on scientific understanding of the processes which govern the performance of its ecological system. Weiss *et al.*, on page 665 of this issue¹, give a new insight into the most important problem of such understanding — the mechanism by which the deep waters of Lake Baikal are constantly supplied by oxygen, keeping them at no less than 75 per cent of saturation², and by which nutrients are delivered from deep waters to the surface to be consumed by planktonic algae.

It is conventionally understood that mixing in lakes in the temperate zone is attributable to water's abnormal variation of density with temperature (decreasing below temperatures of 4 °C). But this underestimates the complexity of deep vertical mixing in many large lakes³, not least the deepest, Lake Baikal. The basis of the circulation study by Weiss *et al.* is the measurement of the distribution of freon in the water column. For many decades, the concentration of freons in the atmosphere has constantly increased.

Because freons are stable

in air and water, the concentration at any particular depth in a lake gives a simple measure of the time since that water parcel occupied the mixed upper layer.

This elegant method involving precise gas-chromatographic analysis has previously been used for ocean waters, although as with lakes, their age has been studied mainly by the tritium method⁴.

Weiss *et al.* find that, unlike other large lakes in the temperate zone which turn over once or twice each year, Baikal's deep waters tend to be much more than one year old, and their age changes with depth. Most remarkably, the near-bottom waters are significantly younger (at 8 years old) than those at intermediate depths (14–16 years old). These data will have to be incorporated into any models of Baikal's ecosystem. The time constant of the response of the water body to perturbations equal to a few decades is an important parameter for proper monitoring and management of the lake, and for the reconstruction of its history and past climate.

Weiss *et al.* do not propose a quantitative hydrodynamic model to fit their data on the distribution of



Satellite image of Lake Baikal, in eastern Siberia, recorded by the Coastal Zone Color Scanner. The plumes at the southeast of the lake, emanating from the brown delta, are from the Selenga River. Northeast of the lake's southern tip is the city of Irkutsk, on the Angara River, the lake's only outlet. On the south coast of the tip is the Baikalsk paper mill (white), a major source of pollution. The whole lake is 640-kilometres long. (Courtesy of Gene Feldman and R. F. Weiss.)

freons, oxygen and nutrients. It is noteworthy that the conditional instability they identify seems to be a mechanism which helps only to extend turbulent diffusion down to a few hundred metres, rather than to full depth. To explain the observed increased concentrations of freons in the near-bottom waters compared with those occurring at intermediate depths, it will be necessary to elaborate models that take into account large-scale convective processes and that involve whole basins in which huge water masses are carried to the very bottom.

The work of Weiss *et al.*, based on data collected during a joint Soviet-US expedition to Lake Baikal in 1988, marks an excellent start to the development of multidisciplinary research sponsored by the Siberian Division of the Soviet Academy of Sciences. In 1988, three international groups made expeditions to Lake Baikal; in 1990, another 25 visited, with participants from 15 countries. The topics covered were diverse — physical limnology, investigation of endemic species by methods of classical and new biology, survey of the capacity of the delta of the Selenga River (see figure) to trap heavy metals and other pollutants, radio-tracer measurements of the rates of accumulation of sediments, seismic profiling and coring of the sediments, search for biologically active compounds in baikalian macro- and microorganisms, studies of aerosols, satellite tracking of seal migrations, and so on.

Last year, two *Pisces* deep-water submersibles belonging to the Academy of Science's Institute of Oceanography probed the lake bed. A large programme involving dozens of institutes and universities from the Soviet Union and United States is planned for investigating the climate history of Central Asia as recorded in Baikal sediments, 6-km thick. The data are necessary for global climate modelling. The Baikal International Center for Ecological Research, a project intended to promote cooperative research, as described in *Nature* two years ago¹, was officially opened in December. One of the first groups to attend this centre will be that of Ray Weiss, members of which are planning to return to Siberia in June to continue their joint Soviet-US study of the lake's deep circulation. □

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Later for the rendezvous

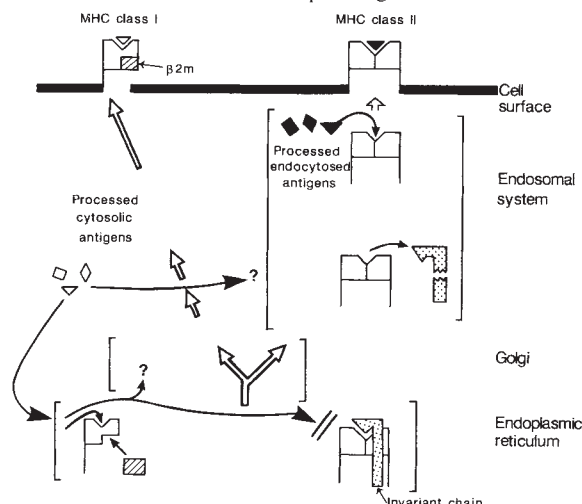
Charles J. Hackett

FOREIGN protein molecules encountered by macrophages, dendritic cells, B lymphocytes or similar antigen-presenting cells generally become processed by unfolding and proteolysis to short linear segments, some of which may fit into the antigen-binding groove of class II major histocompatibility complex (MHC) molecules. Bound antigens are then displayed on the cell surface, allowing T cells with receptors specific for the foreign molecule together with portions of the MHC to be triggered. Although antigens are acquired from outside, antigen processing and association with class II MHC occurs inside the antigen-presenting cell. On page 669 of this issue¹, Peters *et al.* now show that the intersection of antigen and class II molecules may occur well after antigen uptake, in a subcellular compartment having lysosomal characteristics, but short of classical dense lysosomes.

Class II molecules follow an intracellular programme distinct from that of the class I proteins encoded in adjacent regions of the MHC locus. Class I MHC molecules present peptides generated from self or viral proteins biosynthesized within the cell², binding peptides transported across internal membranes (for a review see ref. 3). Association with peptides soon after biosynthesis is evidently a prerequisite for transport of class I MHC to the cell surface⁴, as is the noncovalent association with β_2 -microglobulin (see figure). Class II MHC molecules are heterodimers that become associated with the invariant chain polypeptide soon after synthesis. The invariant chain seems to have two main influences on class II molecules: it makes their peptide-binding site inaccessible⁵, and influences the cellular routing of the complex⁶.

An immunologically critical distinction between the two MHC molecules is that class II molecules intersect endocytosed antigen whereas class I do not. Exogenous antigens are taken into endocytotic vesicles that change in composition and properties as they progress from their peripheral 'early' location to the perinuclear 'late' compartment, towards their intracellular destination of merging with lysosomes (for a review see ref. 8). Endosomes become acidified, and take delivery of proteolytic enzymes and eventually of receptors and enzymes typical of lyso-

somes. It is also possible that, rather than being individual vesicles travelling along this route, endosomes are pre-existing organelles that receive and transmit contents of many individual vesicles along the endocytotic pathway⁹. The proteolytic environment of endosomes seems to be critical to the function of class II MHC molecules, because not only must foreign antigen be processed before it can bind MHC, but removal of the invariant chain requires proteolysis¹⁰. Clearly, MHC molecules must maintain their own integrity in a proteolytic environment, as well as intercept antigen before it is com-



intracellular programmes followed by class I and class II MHC molecules. Class I molecules bind β_2 -microglobulin (β_2m) and processed antigen before transport to the cell surface. Heterodimeric class II molecules on their way to the plasma membrane intersect the cellular endocytotic system, where the invariant chain is shed and processed antigen binds.

pletely degraded to amino acids, if antigen presentation is to be successful.

Where on the pathway of endosomal 'maturation' do MHC class II molecules enter the scene? In recent studies, gold-labelled antibodies viewed in thin section by electron microscopy have been used to locate class II MHC molecules in cellular compartments which have been identified as endocytotic by co-localization of endocytosed antigens and other marker molecules. Although no method as yet can follow a given T-cell determinant throughout the cellular handling processes, immunoelectron microscopy has greatly increased the resolution of structures involved in antigen presentation. Last year, Guagliardi *et al.*¹¹ found by this means that the components needed for antigen presentation — endocytosed foreign molecules, proteolytic enzymes and MHC class II molecules — could be found together in early endosomes within two minutes of the internalization of antigen. Co-localization of the invariant chain,

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5. *Nature* **338**, 193 (1989).

combined with lack of evidence of extensive spontaneous internalization of cell-surface class II molecules, suggested that these are newly synthesized MHC proteins on their way to the surface. The findings implied that class II MHC molecules are present from the earliest contact between antigen and the cellular processing machinery, and may potentially bind antigenic peptides as soon as they become available, shielding them for further degradation^{12,13}.

Such early rendezvous of class II MHC and antigen was not observed by Peters *et al.*¹. Although agreeing that it is newly synthesized class II molecules rather than those recycled from the cell surface that are detected in the endocytotic system, they find that class II MHC proteins in the endocytotic pathway are confined primarily to structures resembling lysosomes; that is, much further along the pathway than early endosomes. According to this picture, endocytosed antigens must run nearly the full gamut of acid pH and endosomal proteolysis before having the opportunity to bind class II molecules, suggesting that antigenic peptides would have to be hardy survivors of the endosomal degradative system. In this regard, Peters *et al.* observed that it took 20–50 minutes for a proteolytically resistant endocytosed molecule to reach the lysosome-like compartments containing class II molecules; meanwhile, the more readily degraded bovine serum albumin molecule was detected there only poorly, implying that proteolysis takes its toll of material moving in to deeper structures.

Clearly, there are labile T-cell determinants that are not presented well from endocytosed antigens¹⁴ or whose presentation is markedly enhanced by inhibition of cellular proteolytic activity^{15,16}. Whether this functional degradation of T-cell determinants observed in biological assays results from a prolonged trip towards class II MHC in an increasingly harsh endosomal environment requires direct information; failure to detect molecules by electron microscopy does not necessarily mean that T-cell epitopes could not be pulled from the digest by MHC molecules. Further, although the lag period of 30–60 minutes needed for endocytosed antigen to become available for T-cell recognition¹⁷ fits neatly with the time taken by antigen to travel to the lysosome-like MHC-containing structures, it is not established that this is the rate-limiting step in antigen presentation. On the other hand, the observation by Peters *et al.* that intracellular class II MHC molecules have half-lives of hours may explain why antigen presentation can persist for hours after cellular protein synthesis has been inhibited¹⁸.

How can the findings of Guagliardi *et al.*¹¹ and Peters *et al.*¹ be reconciled? Information is needed on how results

could be affected by use of different cell lines, or cells cultured under different conditions. The degree of resolution of molecules is also a consideration, but here one would expect that the thawed cryosections used by Peters *et al.* would be more sensitive than the plastic-embedded material of Guagliardi *et al.* In any event, no technique has yet revealed where the association between antigen and class II molecules actually occurs. Experiments addressing functional association — that is, experiments using T-cell recognition — will probably be required in order to resolve this issue.

There are also pressing questions on the related immunology. For instance, is there a way by which endogenously synthesized antigens (for a review see ref. 19) could enter a compartment to associate

with class II MHC, other than by uptake at the cell surface? Are there mechanisms in the class II/antigen presentation system similar to those uncovered for class I, such as molecules which actively translocate peptides to compartments for MHC association, or the need to form a complex with antigen as a prerequisite for transport of MHC to the cell surface? Finally, can the observation that protein antigens are presented by intact antigen-presenting cells faster and at much lower concentrations than expected from studies with isolated molecules²⁰ be explained by how peptide and MHC meet in specialized intracellular compartments? □

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Come in LDEF, your time is up



THE first satellite to be recovered by a space shuttle, the Long Duration Exposure Facility (LDEF) was retrieved by Columbia on 12 January 1990. It is seen here in its last moments in free-fall. LDEF was launched in 1984 to study the effects of cosmic dust and rays. The experiment reported by Fishman *et al.* on page 678 of this issue may indicate the kind of surprises in store for participants in the project. Amongst the gases and ions swept up by LDEF during its mission were prodigious quantities of beryllium-7. Produced by cosmic-ray interactions with air at an altitude of 40 km, ⁷Be has a half-life of only 53 days, so that the authors are at a loss to explain how it came to be trapped by the satellite. □

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Making a submicroscopic hole in one

Charles F. Stevens

COMPLEMENTARY work in three laboratories, reported on page 700 of this issue¹ and in this week's *Science*^{2,3}, has provided the best answer yet to one of neurobiology's central questions: what is the structure that constitutes an ion-selective pore?

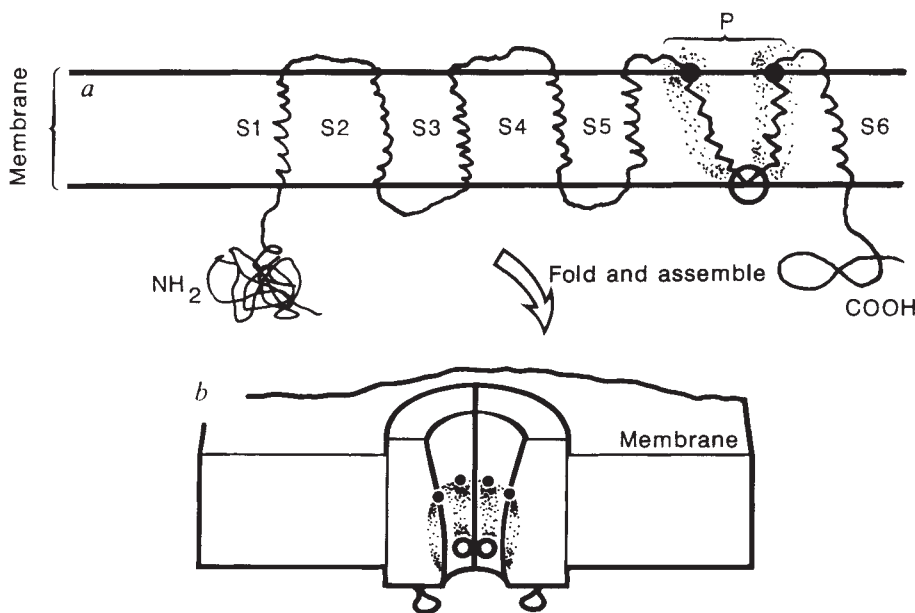
A special class of proteins, called channels, is responsible for the electrical activity of neurons. These channels work by opening and closing the gate that permits ions to flow through a submicroscopic pore. Members of one large subclass of channels have their gate regulated by the voltage difference across the neuron's membrane and are all products of a gene superfamily.

The pores of these voltage-gated channels have the amazing ability to distinguish between ions. For example, the crystal radius of potassium ions is 0.13 nm and that of sodium ions is 0.1 nm, yet various channel types have pores that can tell these chemically very similar ions apart. Potassium channels permit potassium ions to pass through their pore while effectively excluding sodium ions, whereas sodium channels have the opposite selectivity. The structural basis for this selectivity, a phenomenon at the very heart of all electrical excitability, has perplexed electrophysiologists for half a century. Now molecular biological techniques have almost certainly identified the amino-acid sequence that constitutes the ion-selective pore of potassium, sodium

and calcium channels. And the pore is not what some would have expected — it contains no charged residues, and the pore-forming sequence is not particularly hydrophilic.

The voltage-gated channels all have a structure like the one illustrated in the figure. Six transmembrane helices, designated S1 to S6, have been identified (although not yet actually proved to have that structure); the segment of sequence between S5 and S6 is labelled in the figure as P. The recent experiments¹⁻³ identify P as the pore-forming sequence. Although the current evidence directly links P to the ion-permeation pore only in several specific members of the family, the P region surely must have the same function in all members of the superfamily. Because some family members select for sodium ions, others for potassium and still others for calcium, one can anticipate that the specific structural elements responsible for ion selectivity will now be quickly identified.

The first experimental clues that the P region might constitute the pore came from experiments that used site-directed mutagenesis to modify the sensitivity of ion channels to certain toxins⁴⁻⁷. Pharmacological agents have long been known to block the function of ion channels by plugging them up, much like a cork plugs a bottle, and several laboratories had identified mutations in P that alter the



Structure of a voltage-gated channel showing, *a*, one subunit of a potassium channel with six transmembrane helices (S1–S6) and the newly identified pore-forming region (P); ● and ○ are the sites that bind tetraethyl ammonium (TEA) ions from the outside and inside respectively, the second site being part of the selectivity filter. *b*, A channel is assembled from four subunits, two of which are shown here. Four P regions make up the ion-selective pore.

RÉSUMÉ

Headbangers

BLIND, subterranean mole rats truly feel the presence of their fellows, E. Nevo *et al.* report (*Proc. natn. Acad. Sci. U.S.A.* **88**, 1256–1260; 1991). Mole rats of the species group *Spalax ehrenbergi* use a crude but effective method to communicate over long distances — thumping their flattened heads against the walls of their tunnels. The seismic signals so generated travel well and help to mediate the territorial behaviour of the rodents. It has been thought that the vibrations are detected as sounds, transmitted through the bones to the ear. But Nevo *et al.* show that the signals can be detected independently of the auditory system, presumably by mechanosensors near the animals' surface.

Inside out

THE 'Dupal' isotopic signature, found in basalts from ocean islands throughout much of the Southern Hemisphere, is probably derived from deep in the Earth's mantle, according to D. Weis *et al.* (*Geology* **19**, 99–102; 1991). The Dupal anomaly is identified by its isotopically unusual lead and strontium content. The question, since it was discovered by Dupré and Allègre, has been where it comes from. The new results are from the Ninetyeast Ridge, a 5,000-km-long rise below the Indian Ocean. Geochemical sampling shows that the basalts forming the ridge are related to those of Heard island in the Antarctic. If the hot mantle plume currently generating the Heard magmatism was also responsible for Ninetyeast Ridge, which started to form 115 million years ago, it must be deep seated, the authors argue, originating at least at the 670-km-deep boundary between the upper and lower mantle, if not deeper.

Red-blooded reindeer

THE combined efforts of laboratories in Italy, Norway and Finland have brought to light another example of evolutionary fine-tuning in haemoglobin. R. Petruzzelli *et al.* (*Biochim. biophys. Acta* **1076**, 221–224; 1991) have determined the sequence of reindeer haemoglobin, to assist in interpretation of its function. It has a markedly low affinity for oxygen, and its heat of oxygenation is highly exothermic in the deoxy (R) state and close to zero after the conformational switch (T state). This means that oxygen delivery to the peripheral tissues in the winter (ambient temperature down to –40 °C) remains efficient. The cofactor, 2,3-DPG, plays no part in the physiology, for it hardly binds; this is a consequence of mutations at its β -chain site, and also of enhanced competition from Cl[–]. If release of Cl[–] by the R state is endothermic it will tend to cancel the heat of the intrinsically exothermic oxygenation process.

sensitivity of both sodium and potassium channels to toxins. The most compelling of these investigations indicated that the sites noted in the figure (a, right-hand side) interact closely with toxins in the extracellular medium and thus could be near the outside mouth of the pore.

Hartmann *et al.*² used these clues to exploit two different potassium channel clones that, when expressed in oocytes, exhibit different pore properties. The two clones differ in three functional characteristics: conductance of the open channel, and sensitivity to block by tetraethyl ammonium (TEA) ions from the outside and from the inside (the inside and outside TEA-binding sites were known to be distinct). When Hartmann *et al.* transplanted a region of P from the first channel type to the second — the chimaeric channel had nine amino-acid substitutions out of 21 residues — the channel properties followed the transplant without, amazing as it seems, there being other dramatic changes in channel function. Thus, these nine substitutions seemed to alter TEA blocking from both inside and outside, and to change single-channel conductance — just what one would expect if P formed the pore.

If the region of P modified by Hartmann *et al.* really is the pore, then modest structural changes in this region should alter the selectivity to various ions. Potassium channels permit the passage of potassium ions and exclude sodium ions, but they also permit entry of two ions that have crystal radii larger than potassium — ammonium (0.14 nm) and rubidium (0.15 nm). Yool and Schwarz¹ found that two specific amino-acid substitutions within P modified the pore permeability to ammonium and rubidium without changing other properties of the channel. Neither of the substitutions made by Yool and Schwarz included residues altered by Hartmann *et al.*, although they are in the same region.

These first two studies^{1,2} demonstrate that P is critically involved in ion permeation and selectivity, but they are less informative on the question of what amino-acid sequence actually spans the membrane: the changes made by Hartmann *et al.* changed something on the inside because they altered TEA sensitivity from the inside, but substituting nine amino acids might have produced significant structural modifications in neighbouring regions. Yellen *et al.*³ have found, however, that the conservative threonine (T) to serine (S) mutation — a change that Yool and Schwarz found alters ionic selectivity — in the middle of P alters the blocking of the pore for inside, but not outside, TEA. So either P loops from the outside to the inside and back again, forming the pore, or the T-to-S mutation produces some structural changes that propagate to the inside, an extremely unlikely possibility

for such a conservative substitution.

Few studies that employ site-directed mutagenesis have faced the issue of a mutation's sphere of influence — the distance over which structural perturbations propagate — and even crystallography, with its elucidation of frozen structures, cannot always provide a final solution. Nevertheless, the three studies described here convince me that we have the pore: P loops in and out, and forms the structure that permits ions to enter and leave the cell.

What are the next steps? Having identified the structure, we now need to work out how the amino acids are arranged in three dimensions, and how, or to what extent, this structure accounts for ionic selectivity. This is a hard problem which will not yield answers immediately, but we now have a firm place from which to start.

The new observations have at least two general implications. First, the striking thing about the swap made by Hartmann *et al.* is how little other functions of the channel were modified. Their results indicate that one can switch in an entire function, the permeation of a channel, while keeping the bulk of the channel structure unchanged. The P region lies between a pair of introns, so I view the observations as supporting the notion that even quite fancy proteins can be constructed of modules. Second, it was predicted earlier, purely from an examination of the primary structure of channels⁷, that P forms the pore. Unlikely as this might seem, the results summarized here give strong support to the idea that one can deduce unusual aspects of three-dimensional structure from the amino-acid sequence of a membrane protein. Of course, one does not know the limits of validity for such structural predictions — a question which needs intensive study — but this victory by a predictor of structure may worry those who have previously dismissed such efforts out of hand.

When all is said and done, however, what we really need is high-resolution structures of channels. Those of us in the channel business feel that we are doing our part, and we wonder when the structural biologists are going to do theirs. □

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Internal skin-care

SOME chemicals, like the active principle of garlic, many compounds of tellurium, and dimethyl sulphoxide, can cross the skin barrier and enter the body by simple contact. Once inside, they can also come out again by the same route. Garlic fanciers and tellurium chemists tend to smell of their interests, as the odorous substances emerge relentlessly through the skin.

Daedalus is now trying to apply this principle. His first goal is an 'internal perfume'. Taken in pill form, it would break down in the body to a substance which sweated steadily out through the skin. Unlike conventional perfumes, it could not be washed off, but would deliver its odorous message evenly and uniformly for hours or even days at a time. Daedalus points out that many perfumes are derivatives or imitations of the subtle sex attractants of animals, which are of course glandular secretions designed to escape from the creature in just this way. So the principle seems fairly sound.

His second goal is an internal insect repellent. Some trees already have one: they protect themselves from insect attack by exuding hormone-like substances which disrupt the life-cycle of the pests. Many animals are tortured so mercilessly in their natural state by flies, mosquitoes and other insects that they too should have evolved a chemical defence. DREADCO biochemists are visiting the local zoo to take blood or sweat samples from giraffes, antelopes, wildebeeste and so on. They plan to chromatograph their specimens into components, and screen them for activity as skin-passing insect repellants or even insecticides. From the results, they hope to design a human version. In pill form, it will protect its swallower against the hungriest mosquito or most enraged wasp.

But DREADCO's main ambitions in this field depend on the decay of the ozone layer. The rising ultraviolet content of sunlight is spreading skin cancer among sun-loving white-skinned people. An internal sun-blocking pill should be a real winner. Again, nature may have got there first. Chameleons, frogs and many other creatures react to the colours in their environment. Does their camouflage skill extend into the ultraviolet? If so, their skin chemistry might be adapted to human use. Daedalus imagines some photosensitive chemical which circulates harmlessly inside the body, but is vulnerable to ultraviolet light when it traverses the skin capillaries. Such radiation photolyses it and binds it to the skin at that point as a substance which itself absorbs ultraviolet strongly. It will thus migrate to, built up in, and protect the exposed regions of the skin. A little extra biochemical cunning might even arrange for it to confer a becoming tan.

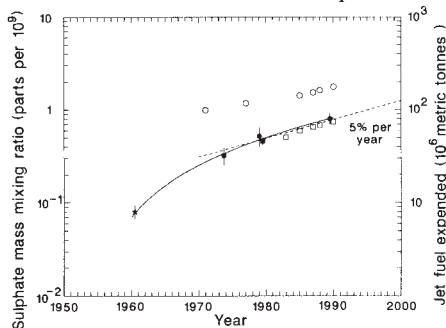
David Jones

Aircraft sulphur emissions

SIR—The background level of non-volcanic stratospheric sulphate aerosol may be increasing at a rate of about 5 per cent per year¹. This increase is believed to be due to an increase in sulphuric acid in the stratosphere, possibly derived from sulphurous gases emitted naturally and anthropogenically at the surface. The most likely candidate is carbonyl sulphide, which is relatively inert in the troposphere, where it has been observed at the 500 parts per 10¹² by volume level since 1976, and is mainly decomposed by ultraviolet radiation present above the ozone layer². But although carbonyl sulphide may be a major contributor to stratospheric sulphate, its concentration does not appear to be increasing (R. A. Rasmussen, personal communication) adequately to explain the apparent increase in the background stratospheric sulphate. Model calculations³ indicate that an increase in carbonyl sulphide emissions by a factor of five would increase the stratospheric sulphate level only by a factor of about three. Thus, to explain the sulphate doubling time of about 15 years, carbonyl sulphide level would have had to double in the last 9 years and now be in excess of 1 parts per 10⁹ by volume, which it clearly is not. Additional sources must therefore be responsible for the increase. One of these could be sulphur emitted in the exhaust of jet aircrafts flying in the 11–12-km region.

Major volcanic eruptions, which explosively inject sulphur dioxide into the stratosphere, are well known to increase the stratospheric sulphate level for years following an eruption⁴. Stratospheric sulphate measurements not affected by volcanic influences are rare (see figure for the data that exist)^{1,5,6}. Also shown in the figure is jet fuel consumption, which has been increasing at a rate of 5 per cent per year during the 1980s, similar to the background sulphate levels. The fact that these two variables appear to be increasing at the same rate has no significance unless one can show that a physical mechanism links the two and that the sulphur budget is in order. One feature which would make an upper tropospheric/lower stratospheric small particle source, as opposed to a surface-based gaseous source, favourable for production of background stratospheric sulphate is the coagulative nature of the growth process wherein the large particles of the sulphate layer would benefit through larger coagulation coefficients (only the larger particles appear to have increased⁷). Another is that carbonyl sulphate models³ predict a maximum in the sulphate layer at altitudes of 25–30 km (above the ozone maximum where adequate ultraviolet is present) whereas background observations indicate altitudes of 20–25 km for the sulphate maximum.

The measurement of jet aircraft effluents in the 11–12-km region is complicated by the large natural aerosol component. Aircraft flying in the relatively clean stratosphere do create sulphuric acid aerosol, as was demonstrated in 1976 when a balloon equipped with aerosol particle counters inadvertently penetrated the exhaust plume of an SR-71 reconnaissance aircraft at 23 km, 18 hours after passage over Laramie, Wyoming⁷, forming a dense cloud of very small (radius of about 0.02 μm) sulphuric acid droplets in the wake of the aircraft. The sulphur mass



The measured mass mixing ratio of sulphate at the maximum in the stratospheric sulphate layer at about 20–22 km altitude at northern midlatitudes during volcanically quiescent periods (solid symbols) and the annual consumption of kerosene jet fuel (open symbols) versus time since 1960. The data of Junge *et al.*⁵ and Hofmann¹ were obtained from balloon soundings and that of Sedlacek *et al.*⁶ were obtained during high altitude aircraft sampling. The fuel data are from the International Energy Agency, Paris⁸, the 1990 data are Federal Aviation Administration (USA) projections. The dashed line represents a 5 per cent per year increase. ★, Sulphate (ref. 5); ■, Sulphate (ref. 6); ●, Sulphate (ref. 1); ○, Worldwide jet fuel consumption; □, US jet fuel consumption.

observed in the aerosol was consistent with the fuel expended and a sulphur fuel content of about 0.1 per cent by weight (the internationally specified maximum is 0.3 per cent).

The current mass concentration of the background stratospheric aerosol is about 0.1 $\mu\text{g m}^{-3}$, or about 0.025 $\mu\text{g m}^{-3}$ of sulphur for the approximately three-fourths H_2SO_4 /one-fourth H_2O composition¹. For a global layer 5-km thick and a lifetime of 1 year typical of volcanic stratospheric aerosol, a sulphur source of $6.25 \times 10^7 \text{ kg yr}^{-1}$ is required to sustain the background aerosol. Worldwide jet fuel consumption was $153 \times 10^9 \text{ kg}$ in 1987 (ref. 8) which, for a sulphur concentration of 0.1 per cent, amounts to a sulphur source of $15.3 \times 10^7 \text{ kg yr}^{-1}$. About 1/6 of the Northern Hemisphere air traffic takes place directly in the stratosphere (S. Wofsy, personal communication), giving a direct sulphur source of about $2.6 \times 10^7 \text{ kg yr}^{-1}$. Of the remaining $12.7 \times 10^7 \text{ kg}$

yr^{-1} , about 40 per cent ($6.1 \times 10^7 \text{ kg yr}^{-1}$) is emitted above 9 km (ref. 8). Only a fraction of this would enter the stratosphere through dynamical processes. This amount, which is latitude and model dependent, may be as large as a quarter, and this would result in a total sulphur source of about $4 \times 10^7 \text{ kg yr}^{-1}$. As this value is about 65 per cent of the required source strength, the jet fleet appears to represent a stratospheric sulphate source to contend with.

Worldwide airline traffic is expected to double by the year 2005 (ref. 9) — a doubling time of 15 years. If the stratospheric sulphate mass also doubled at this rate, it would reach the levels observed after the major 1982 eruption of El Chichón⁴ (about $2 \mu\text{g m}^{-3}$) in about 65 years. Stratospheric temperature and ozone perturbations resulting from this increased aerosol burden could be expected¹⁰. Because there is not enough airspace to accommodate the increased traffic, the use of the stratosphere for commercial aircraft is being considered. This would greatly aggravate the situation, so that if stratospheric aircraft were to be developed, they would have to burn fuel that is essentially sulphur free.

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An electrostatic attraction

SIR—There are many discussions of possible hazards from electric and magnetic fields emanating from television and computer screens. My measurements suggest that natural radioactivity and high electrostatic fields are more likely to be the cause of various common complaints from computer operators. Radiation levels as high as 6,000 alpha disintegrations per minute per 100 cm^2 area of viewing-screen area have been detected on common computer displays and television screens. I show here that the phenomenon is caused by the electrostatic deposition of charged radon progeny onto the faces of viewing screens.

Radon-222 gas is formed by the radioactive decay of naturally occurring uranium-238. Most of the radon in indoor air comes from the first metre of under-

lying soil and rock and enters living areas by diffusion, convection and ventilation. Radon-222 decays through several alpha and beta decays into various radioactive isotopes of polonium, bismuth and lead. Radon progeny are usually charged positive ions which readily attach to dust and aerosol particles in the air. These charged clusters can drift in the high electric fields surrounding the faces and bezels of computer video display terminals (VDT) and television sets. This electrostatic pumping action can collect and concentrate the radioactive radon progeny from a very large volume of air surrounding the viewing screen resulting in a high level of radioactivity on the glass faceplate and surrounding bezel. The electrostatic pump is continuously being fed by convection and ventilation air currents in the room collecting the radon progeny from large air volumes. Rough calculations indicate that as many as 10^7 radioactive progeny can be collected per 100 cm^2 of screen area before the screen is fully discharged. Measurements using a common VDT verify that these total integrated activities were achieved in several experimental runs. Television sets and VDTs in normal use always show alpha activities well above background activities of other objects in the nearby environment (table surfaces, walls, metal plates, glass plates). The maximum activities on television sets and VDTs occur shortly after they are turned off. When turned off, the face falls to a large negative voltage and becomes an efficient collector of positive ions. This pumping action can continue for many hours if the air is dry and the main source of air ionization is from the radon background.

Measurements were carried out in various rooms of our one-storey building

in Santa Clara, California. The radon levels in different rooms were measured with a Honeywell At-Ease radon monitor which displays the current radon level (last 12 hours) and the average level (average since last reset) in units of pico-curies per litre. The monitor has indicated current levels between 0.9 and 3.9 with an average of 1.7 over 90 days. The alpha activity was measured with a 40-cm^2 silicon semiconductor alpha detector followed by the usual charge amplifier, shaping amplifier, threshold discriminator (3-MeV threshold) and electronic counter. The system energy calibration was determined using a plated Am-241 alpha source (5.78 MeV). The detector is taped to various locations on the glass faceplate and/or bezel for activity measurements. The data are normalized to 100 cm^2 and 4π geometry as recommended for reporting surface activities. Measurements were made on several types of common micro-computer VDTs and colour television sets. Colour monitors show the highest levels of activities, which is expected as these monitors use the highest voltages in the cathode ray tubes. Alpha activities generally increase with the current radon concentration but are highly variable on a day-to-day basis. The highest level observed to date was 9,000 $\alpha\text{-d.p.m.}$ 100 cm^2 measured on a colour computer VDT operated in a closed front office. The radon level was not measured in the room, but the radon level in the more open part of the building was at 3.9 pCi l^{-1} at the time of the measurements.

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New image

SIR—Daedalus has hit the mark again in his article about light-pulse imaging of body tissues (*Nature* **348**, 290; 1990). Measurement of the transmitted ray does provide imaging of body tissues (*Photon Migration in Tissues*, ed. B. Chance; Plenum, 1989). The technique works for small objects such as fingers, hands and small animals and, with difficulty, for mammography.

The difficulty is that the early component of the light pulse that takes a direct path constitutes a smaller and smaller fraction of the light as one goes from fingers to arms to heads to chests. In this case more people are interested in what is termed 'photon migration': a random walk process (Lord Rayleigh *Phil. Mag.* **37**, 321; 1919). If larger objects are to be probed, for example the head, heart and liver, it is clear that quite different rules of light travel will be applied to photons which migrate from the input position to

the output position along optical paths that are 5–10 times longer than the direct path. The advantages of this photon-migration technique is that many photons travel these devious pathways. Luckily, they migrate in random walk patterns with a rather good mean directionality; the random walk pattern is highly sensitive to the presence of absorbing objects. In fact, migrating photons allow us for the first time to see behind absorbing objects. Many algorithms for image formation are being studied and some appear quite feasible.

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HIV infectivity

SIR—Bourinbaier calculates¹ the amount of p24 gag protein that corresponds to a given number of HIV virions, and discusses how this might be relevant to HIV infectivity *in vitro*. The gag protein content of a retrovirus was first reported in 1965 to be $5 \times 10^{10} \text{ g}$ (ref. 2).

We suggest, however, that measurements of the total amount of viral protein (p24 or gp120) in HIV cultures or in the plasma of HIV-infected individuals are of very limited value for estimation of the number of infectious particles present. Much of the viral protein secreted from HIV-infected cells is non-particulate, and the proportion of (for example) p24 in virions is a function of the viral genotype and the age of the culture³. In extreme cases, less than one per cent of the total p24 and gp120 present is in virions³. Furthermore, many of the intact virions that exist are non-infectious³. The calculations made by Bourinbaier are thus oversimplistic¹.

In sera from HIV-infected individuals measurement of p24 content is of even less value for estimation of the number of virions present. Commercial ELISA kits for p24 antigen rely on its capture by anti-gag antibodies. Binding of the kit antibodies is interfered with by serum anti-gag antibodies to a variable extent dependent on the antibodies in the kit and the individual serum assayed. Absolute quantification of serum p24, as opposed to its detection, is therefore difficult. Only limited correlations between serum p24 levels and the amount of infectious virus present are found^{4,5}.

Notwithstanding the nonlinear relationship between gag protein content and the number of infectious virions present, we agree on the need for better characterization of parameters influencing HIV infectivity *in vitro*³.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

Necessary precautions

Richard Davenport-Hines

A History of Contraception: From Antiquity to the Present Day. By Angus McLaren. Basil Blackwell: 1990. Pp. 275. £25, \$36.95.

CONTRACEPTION has been as perennial a human need as sex itself. The ways in which it has been practised, together with the social, religious and legal inhibitions which have been placed on it, are central to human experience through the ages, yet have hitherto been largely erased from the historical record. Just as almost none of the modern films and television programmes that feature acts of sexual intercourse ever refer to the contraceptive precautions which the characters presumably take, so historical attempts at fertility control are mostly unmentioned. Professor McLaren's scholarly, comprehensive but sprightly and readable *History of Contraception* is therefore a major piece of historical reclamation and discovery which will interest sociologists, historians and social anthropologists as well as less specialist readers.

He draws on a wide range of demographic, medical, literary, religious and philosophical sources across 2,000 years, and writes lucidly with an appropriate touch of humour. He is mercifully free from the domination of French doctrinaires like M. Foucault. Previous books treating the history of contraception have tended to offer an ethnocentric and even male supremacist view of the subject, in which women are depicted as the passive beneficiaries of the sudden triumph of twentieth-century scientific reason over fertility, ignorance and religious superstition. In fact, as McLaren shows with a mastery of his material that never palls, there have been attempts at fertility control since the era of the Greek city states. The emphasis that he gives to women's experience of contraception, and to women's own efforts at fertility control before the twentieth century, is particularly sensitive, shrewd and salutary. Sexuality has always been "an independent variable" in history, he writes; the continuous, richly complex ways in which reproductive decision-making has been perpetually entangled in a web of economic, social, cultural and gender relations makes a fascinating tale which goes to the heart of human behaviour.

McLaren presents much interesting material on such subjects as condoms or herbal and chemical contraceptives (of varying efficacies), but one of the clearest themes to emerge from his book is the persistent recourse to abortion as the only reliable way of regulating fertility. "The proper thing to do is to limit the size of each family, and if children are then con-

ceived in excess of the limit so fixed, to have miscarriage induced before sense and life have begun in the embryo" — so recommended Aristotle. Throughout subsequent ages the practice seems to have

Fearsome 1930s contraceptive devices.

been unexpectedly widespread and legitimated. In Europe (from which most of the evidence of the book is drawn) there was a traditional notion that, until animation

took place, married women were entitled to seek to restore their menses, although there was always more hostility to abortions necessitated by the results of fornication. But generally, until the nineteenth century, "many insisted on viewing abortion as simply one more step on a continuum of fertility-controlling practices".

It might seem that there is not much in the way of description or analysis to be written about the miserably unsatisfactory and anxious act of *coitus interruptus*. But McLaren is no less illuminating when he assesses the prevalence of this contraceptive technique in Czechoslovakia in the 1970s than when he quotes a poetic description of withdrawal written in the seventh century BC by a mercenary from Paros. Throughout he relates this attempt at fertility control to the different types of society in which it was practised. Although McLaren's interpretative synthesis is in most respects complete, in writing about *coitus interruptus* he overlooks the influential body of medical and quack opinion which (in Britain from the seventeenth to the early twentieth centuries) urged men to make penetration as quick as possible to avoid venereal disease, and which particularly warned that a woman was more likely to transmit such an infection the nearer she approached to orgasm herself. Withdrawal was widely practised as a prophylactic against venereal infection as well as a contraceptive technique. As Lord Rochester described in his poem 'The Debauchee',

*I send for my whore, when for fear of a clap,
I dally about her, and spew in her lap.* □

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Informational school

Gunther S. Stent

The Emergence of Bacterial Genetics. By Thomas D. Brock. Cold Spring Harbor Laboratory Press: 1990. Pp. 346. \$55.

MOLECULAR biology became a distinct discipline in 1953, upon the marriage (brokered by Watson and Crick's discovery of the DNA double helix) between two formerly single bodies with separate research agendas. One of the newlyweds was the structural school, concerned with the three-dimensional form of living molecules. Its experimental material consisted mainly of crystallized biological substances, which were submitted to X-ray diffraction analyses. The other was the informational school, concerned with the transmission and expression of hereditary information. Its experimental material consisted mainly of bacteria and bac-

terial viruses, which were submitted to genetic analyses.

Thomas Brock's *The Emergence of Bacterial Genetics* provides an extensively documented history of the rise of the informational school. Brock has taken the standard chapters of textbooks on the genetics of bacteria and their viruses — classical genetics and bacteriology, mutation, conjugation, bacteriophage, lysogeny, transduction, transformation and gene expression — and expanded them by adding direct quotations from original research publications and presentations of experimental details. He also quotes and summarizes material he found in archival holdings of letters and private papers of some leading members of the informational school and reports on consultations and correspondence with many people who have personal knowledge of the developments covered.

Thus this book offers newcomers to bacterial genetics a wealth of experimental data, as well as peeks behind the scenes. This is especially true of the work of Joshua Lederberg, the discoverer of

bacterial conjugation and virus-mediated genetic transduction, whom Brock has chosen as the hero of his story. For instance, Norton Zinder is quoted as saying that the bacterial strains with which he and Lederberg discovered genetic transduction were smuggled into the United States from Sweden by diplomatic pouch. Even veteran bacterial geneticists may find details of which they had not been previously aware.

Unfortunately, it is hard to be enthusiastic about Brock's book, despite its voluminous historical coverage. There are incoherences in the text, of which I give one example that happens to be of personal concern to me. It is pleasing that Brock takes up the controversy that arose some years ago from my categorizing as premature Oswald Avery's 1944 discovery that the transforming principle is DNA. Brock rejects my claim that it was several years before Avery's discovery made an impact on genetic research, finding that "although [M.] Delbrück and the 'phage church' may have resisted biochemistry and DNA, numerous biologists and biochemists accepted [Avery's discovery] immediately". But after devoting four pages to telling how several important geneticists, especially Lederberg, immediately appreciated, or were inspired by Avery's discovery he concludes that "the real point is that most people discussing transformation were not so convinced of its importance that they began to work on it". Moreover, "although transformation may have provided the original motivation [for Lederberg], it did not help him in his actual work". So maybe I was right, after all, in arguing "that geneticists did not seem to be able to do much with the ... connection between transforming principle and DNA"? No, because "many have argued otherwise". Without telling what those many have argued otherwise, Brock finally refers to H. V. Wyatt's documentary proof of the alleged prematurity, by noting that "*Advances in Genetics*, a major review annual edited by M. Demerec, contained no mention of DNA in the index through 1956".

Brock does not hesitate to hand out demerits and finds faults in others that he manifests here himself. For example, he says that he needs to correct the accounts of "Griffiths [discovery of the transformation phenomenon] made through the years by authors who have not read the original". How does he know that they have not read the original? Presumably because they would not have misrepresented it if they had. From that reasoning it would appear that Brock has not read Cyril Hinshelwood's "widely read and frequently misunderstood" *The Chemical Kinetics of the Bacterial Cell* (Clarendon Press, Oxford, 1946). Because if he had, he would surely have qualified his righteous dismissal of Hinshelwood, whose anti-darwinian, pro-lamarckian "ideas received deference when

they should have received short shrift". He would have pointed out that Hinshelwood put forward the kind of kinetic flip-flop circuit that turned out to explain the pseudohereditary maintenance phenomenon in *Escherichia coli* galactosidase induction and to account for some cases of cell differentiation. If Brock had read Erwin Schrödinger's *What is Life?* (Cambridge University Press, 1944), would he have written that it was anticipated 30 years earlier by L. T. Troland's proposal that "genetic enzymes must be identified with the nucleic acids"? Perhaps he confuses Schrödinger with Avery. He also repeats the canard that Schrödinger's book was "quickly outmoded", when, in fact, its highly original proposal of a genetic code proved to be one of the great biological ideas of the century.

Brock can hardly be said to be fair in his treatment of Jacques Monod and his circle. He writes that most of Monod's colleagues "had short memories or were ignorant of Monod's early work" without documenting this allegation. He asks why Monod ignored four key ideas put forward by Lederberg in a 1951 paper and yet later incorporated these ideas into "the Monod canon". He thinks that "although some chauvinism may have been involved", Monod was also prejudiced in favour of physiological over genetic evidence. But he does not demonstrate that Monod, born of an American mother, was actually a chauvinist. Nor does he show how even a person receptive to genetic evidence could have culled these ideas from Lederberg's text without resort to deconstructionist hermeneutics not yet available in the 1950s. Anyhow, according to Brock, Monod only cited the work of others "when it suited his purpose, but often conveniently ignored it". How he came to know what was in Monod's mind Brock does not say.

Although Brock hopes that "this book will be of interest to both scientists and historians", his text is so short on explanations of technical terms that persons not previously familiar with the subject are going to have a hard time reading it. Their interest may also be diminished by Brock's philosophical platitudes, such as "history is often written as if it moves inexorably in a single direction. However, life does not work that way, and there are numerous false starts and incompletes." I would recommend that they first read Horace Judson's *The Eighth Day of Creation* (Simon and Schuster, New York, 1979). If, after they have acquired a sophisticated overview of the subject from Judson, they wish to learn further details about the history of the informational school, there would be no harm in their turning to *The Emergence of Bacterial Genetics*. □

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Greater genius

Geoffrey Tweedale

John von Neumann and the Origins of Modern Computing. By William Aspray. MIT Press: 1990. Pp. 376. £31.50, \$35.

ASK anyone who were the greatest figures in computer history and the names of Charles Babbage, Alan Turing and John von Neumann will inevitably recur. Of the trio, only von Neumann (1903–1957) lacks a biographer, even though in terms of his actual impact on the development of the computer, he was easily the most influential. (Hence the term, 'von Neumann' machine, which is often used to describe the modern electronic digital computer.)

As one of the most brilliant and precocious scientists of the twentieth century, the main outline of von Neumann's life is already well known. Born in Hungary and educated in Germany, von Neumann had already made major contributions to quantum mechanics and mathematical logic, before taking up a professorship (and then American nationality) at the Institute for Advanced Study (IAS) at Princeton in 1930. He therefore came to computing relatively late in life, largely as a result of the Second World War, when the demand for greater computational power for projects such as the development of the atomic bomb drew von Neumann into the field of calculation. Henceforth, it accounted for a major part of his time. Von Neumann, it seems, was unable to work in any field without leaving the characteristic imprint of his logical and wide-ranging mind, and computing was no exception. His involvement with government-funded projects produced his highly influential report of 1945 on the Electronic Discrete Variable Computer, which was the virtual blueprint for the stored-program digital computer. His reputation and influence greatly raised the standing of the emerging computer profession and allowed von Neumann himself to construct one of the earliest machines within the cloistered confines of the IAS at Princeton in the early 1950s. It sparked research into a variety of scientific fields and was widely copied. By his own example, von Neumann also ensured that technical knowledge about the computer was placed in the public domain.

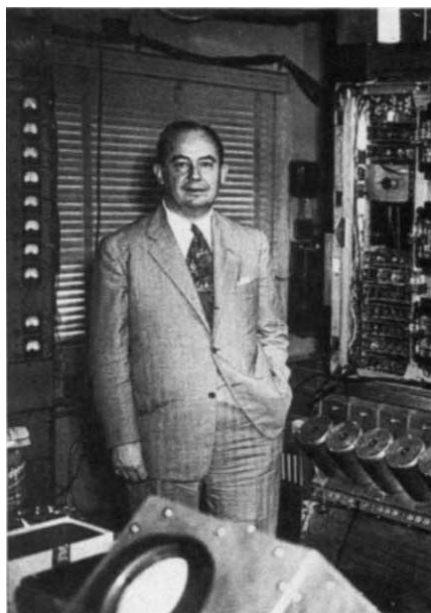
But as William Aspray demonstrates in this book, what made von Neumann so notable (aside from his greater genius) amongst his fellow pioneers was that his work extended well beyond the design and construction of computer systems. Like Turing, von Neumann regarded computers only as a means to an end; in other words, it was computer applications that provided the greatest stimulus to his

talents. Half of the book is therefore devoted to a detailed treatment of von Neumann's work in this sphere.

Von Neumann was one of the first to realize that new mathematical methods would be needed to use the computer effectively and he helped set out the research agenda for computer-orientated numerical analysis. At Princeton, he also established a meteorology project, which helped prepare the first computer methods for weather forecasting and led to the production of daily numerical forecasts. Naturally, he applied the computer to various scientific problems as the opportunity arose and this allowed him to make contributions in fields as diverse as atomic physics, fluid dynamics and traffic simulation. More importantly, von Neumann was fascinated by the analogy between the digital computer and the human brain. Almost singlehandedly he blazed a trail into the theoretical field of information processing systems, or automata, and introduced such themes as learning, reliability of systems with unreliable components, self-replication, and the problems of memory and storage capacity in biological nervous systems. This work, which was tragically and prematurely ended when von Neumann died of cancer in 1957, remains central to modern concerns with parallel processing and artificial intelligence.

Because von Neumann was "violently anti-Communist . . . and more militaristic than most" (his own words), not all of his energies were directed towards constructive ends. It was in America's growing military-industrial complex that he found his true spiritual home and much of his later work involved the development of bombs and delivery systems. Yet, in contrast to Andrew Hodges' recent biography, *Alan Turing: The Enigma of Intelligence*: Burnett Books/Hutchinson, London, 1983 and Steve Heims' study, *John von Neumann and Norbert Weiner: From Mathematics to the Technologies of Life and Death*: MIT Press, Cambridge, 1980, the political, military and economic

dimension are only lightly sketched here. Only a brief final chapter looks at von Neumann as scientific consultant and statesman and there is no discussion of von Neumann's deeper motivations. On the other hand, this book is far better documented than Heims' — Aspray has clearly researched far more archives than any other writer — and it is not intended as a full-scale biography. Within its narrower focus it succeeds admirably and is a



Von Neumann — brilliant and precocious.

major addition to the MIT Press's excellent series on the history of computing. The author's extensive knowledge of the subject enables him to bring new and interesting insights into every aspect of von Neumann's work. He allows us to see the true scale of von Neumann's achievements in computing for the first time and firmly establishes his reputation as the most influential computer pioneer of all time. □

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Beating disease

Roy Porter

Disease, Mortality and Population in Transition: Epidemiological-Demographic Change in England since the Eighteenth Century as Part of a Global Phenomenon.

By Alex Mercer. *Leicester University Press/Columbia University Press*: 1990. Pp. 262. £45, \$69.

How do we explain the great modern rise in population, the taming over the last few hundred years of once terrifying diseases such as typhus and typhoid, and the leap in life expectancy? Traditionally, all these

are put down to the triumphs of scientific medicine. Then, in a series of influential works, Professor Thomas McKeown of the University of Birmingham threw a gigantic spanner in the works. Mortality from conditions such as tuberculosis, he insisted, was on the decline long before effective medical treatments were developed. Indeed, in earlier centuries, medicine itself had all too often been counter-productive — thus hospitals were 'gateways to death'. So what had defeated the empire of disease? The explanation, according to McKeown, lay in rising standards of living, and above all, improved nutrition.

Alex Mercer is one of numerous epidemiological demographers convinced

that the McKeown thesis does not hold water. For one thing, Mercer argues, medicine deserves more credit than McKeown allowed. The development by the medical community first of inoculation and then of vaccination helped conquer smallpox, after the plague's disappearance the most fearsome epidemic disease in Britain. And then the adoption of what we may, in the broader sense, call preventive measures — improved personal hygiene, urban clean-ups, purer drinking water, better waste-disposal, the isolation of sufferers from contagious diseases — initiated that long retreat of the major water- and air-borne infections visible from mid-Victorian times, probably independently of any demonstrable enhancements in diet. And this story of general health and environment improvement, Mercer notes, may hold some lessons for action nowadays in the Third World. Although hi-tech medicine may not be appropriate or affordable, improvements in health need not wait upon general economic progress: elementary public health and personal hygiene initiatives can achieve in Africa today what they accomplished in Manchester and London last century.

Of course, all was not pure gain. The age of communicable diseases was succeeded not by radiant health but by rising incidence of chronic and degenerative conditions, above all cancers and circulatory diseases; and Mercer offers interesting speculations upon possible epidemiological linkages between acute disorders and noncommunicable diseases with multifactorial aetiologies — for instance, cancer as, in some sense, the 'successor' of tuberculosis. Nevertheless, Mercer's analysis provides a credible corrective to McKeown's excessively negative reading of the role of medicine.

The problem is that Mercer adds little new information to a now familiar anti-McKeown line that has emerged during the last decade. Indeed, he fails to integrate any substantial contributions to the case, for instance Linda Bryder's and F.B. Smith's books on tuberculosis, both of which appeared nearly three years ago. Nor, in some cases, does he fully get to grips with the real ambiguities of the evidence. Recent research, above all that of Roderick Floud, has demonstrated that the English grew taller during most decades of the nineteenth century. This suggests better diet and, by inference, better resistance: maybe there is life in McKeown's nutrition theory after all. Mercer offers a workmanlike, if not especially readable, survey of a key debate. We have not yet heard the last word. □

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Nasty physics

Daniel J. Kevles

At the Heart of the Bomb: The Dangerous Allure of Weapons Work. By Debra Rosenthal. Addison-Wesley: 1990. Pp.244. \$18.95.

THE activities that make the American nuclear arsenal are carried out at nine major facilities in the United States, but the key activities — the conception and design of the weapons — are conducted in three places: at the Livermore National Laboratory, in California, and at the Los Alamos National Laboratory and the Sandia National Laboratories, both in New Mexico. Scientists and engineers at Livermore and Los Alamos design the explosive guts of nuclear warheads — called 'physics packages' — and their counterparts at Sandia, which is located inside the Kirtland Air Force Base, convert the packages into weapons, imbedding them in configurations that will deliver and detonate them. Each of the laboratories is a GOCO, a government-owned, contractor-operated enterprise. The American Telephone and Telegraph company runs Sandia for a dollar a year; the University of California manages Livermore and Los Alamos, for some six to seven million dollars a year, a tiny fraction of the university's eight-billion-dollar annual budget. Each laboratory employs 8,000 people, all civilians, all directly or indirectly involved in the creation of weapons of mass destruction.

In 1984, Debra Rosenthal, then a junior member of the political science faculty at the University of New Mexico, set out to discover how the people at Sandia and Los Alamos live with such work and what sense of social responsibility they have about it. Ultimately, she conducted 260 hours of interviews with 85 people, about half from each facility, thereby obtaining the material that forms the base of *At the*

Heart of the Bomb. Rosenthal holds confidently that the people she describes "represent the range of opinion within the weapons laboratories, if not the precise distribution." Perhaps, perhaps not: she appears to have made some attempts to meet the requirements of survey research, but the attempt was not very vigorous (more than one third of her interviewees volunteered). Nevertheless, she got people to talk, and she straightforwardly reports what they said, framing their attitudes in the context of their personal backgrounds, experiences and apprehensions of the Cold War. The result is a fascinating and revealing portrait of the hearts and minds of a select group of people who derive their livelihood — and more — from the core of the nuclear weapons culture.

Outwardly, there is little distinctive about Sandia employees, many of whom live in Albuquerque, or about Los Alamos staff, whose community is not much different from any small town that is dominated by a single industry. Rosenthal writes of Los Alamos, whose wartime citizens included J. Robert Oppenheimer, Edward Teller and Enrico Fermi: "No egghead eccentrics are visible on Trinity Drive nowadays. In their stead are normal-looking fellows in sensible cars who obediently stop for panting joggers in warm-up suits." A kind of regular, workaday quality characterizes the bomb-making enterprise. Some of Rosenthal's respondents acknowledged that their work has unpleasant features but preferred to shrug them off, pointing to how their jobs provide salaries or research funds that were too good to pass up. A man called Walt had decided in junior high school that designing weapons would be a safe haven from being sent off to war and returning in a body bag. He was just a "voyager in space", he told Rosenthal, adding, "I just happen to be in the century, and I'm trying to get through it. I really have no control over it."

A number of scientists at Los Alamos and even Sandia do basic research that is unrelated or only indirectly related to bomb design. Although Rosenthal neglects to provide either an overview of the research programmes at either facility or how the scientific staff is distributed among them, she reports that the nuclear weapons groups are relatively small. Elsewhere in the laboratories, their members are known as bombheads, scientists who identify with the weapons, get caught up in their fascinating technical details. Members of the bomb design branch at Los Alamos call their research 'nasty physics', but absorbing physics it is nevertheless. Unquestionably, weapons design poses scientific challenges that are sufficiently compelling to quell moral doubts.

Some of the weapons scientists

approach their work with untrammelled pleasure — some might say unseemly joy — thrilled by the scientific chase and driven by a hunger for Promethean accomplishment. Karl, a master of thermonuclear weapons design, boasted to Rosenthal of "nine Nevada shots with my name on them." Although he had seen the films and the instrument reading of test explosions, he said that he still wanted to "feel the heat", adding, "This is the most there is; this is the closest you get to playing God." Karl noted that "stars and bombs are a lot alike, but you don't get to design a star and see if it'll go supernova when you want it to."

Karl likes to tell peace activists that "if God hadn't loved bombs, he wouldn't have invented [the] Japanese", but Karl's callous defence of nuclear weapons work appears to be anomalous. Rosenthal's interviewees advanced a variety of justifications for their work, most commonly a secular concern for national security, a religious anti-Communism, or some combination of the two. Virtually all held to the position that they were no less moral about nuclear weapons or more responsible for them than the president, the congress and the American people, who by and large have wanted such arms, supported by policy and taxes the system that produces them, and benefited from the protective umbrella that they provide.

Only eight of Rosenthal's respondents said that they had ever wanted to leave their jobs. Even then, the goads to departure were often not moral objection but dissatisfaction with the irritations of security rules, the bleakness of the Sandia ambience, or the scientific isolation that comes with involvement in classified research. Still, reasons of conscience have taken some from the laboratories, including Tom Grissom, a 15-year veteran of Sandia, who in a six-page essay written to explain himself to his children and friends, declared, "I prejudge our guilt to be the same as that of all the good Nazis who obediently followed orders." Rosenthal seems to sympathize with Grissom's point of view, neglecting to analyse the numerous differences, moral and otherwise, that distinguish nuclear weapons designers from, say, Adolf Eichmann. However, she does seem to recognize that the problem Grissom confronted resides rather more in the machinery of the arms race than it does in any individual moral complicity. She notes that Grissom's simple personal solution appears "hopelessly idealistic" to the workers in the laboratory, people who "feel themselves enchanted by and trapped within a huge mechanism that promises to function smoothly with or without them." □

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New in paperback

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Deep-water renewal and biological production in Lake Baikal

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The physics of mixing in deep temperate lakes is strongly constrained by the existence of a temperature of maximum density for fresh water, and by the pressure dependence of that temperature. The world's deepest lake is well suited to the study of such deep-water renewal processes, and also to the determination of the rates of renewal using time-dependent chemical tracers. The mean rates of biological recycling of oxygen, carbon and nutrients for the entire lake can then also be determined.

LAKE Baikal, in the great Baikal Rift of eastern Siberia (53° N, 108° E), lays claim to many superlatives: the world's deepest (1,632 m) lake, the world's largest volume of fresh liquid water (23,000 km³), the home of many unique animals and plants, a region known for its remote isolation and pristine beauty, and most recently a major focus for a growing Soviet environmental movement concerned about the effects of industrial development and pollution¹⁻³. One of the most fundamental pre-requisites to understanding any deep lake as an integrated physical, chemical and biological system is its rate of deep-water renewal by exchange with surface waters, or 'ventilation'. This process controls the internal redistribution of water properties, and so controls the time-dependent response of the lake to its own chemical and biological processes, as well as to natural and anthropogenic external perturbations. For example, as discussed below, a lake's biological productivity, and hence its entire food web, is limited by the rate at which vertical exchange supplies nutrients to the surface waters from below. The response of a lake to changes in atmospheric or riverine inputs is similarly dependent on the mechanism and rate of vertical exchange between surface and deep waters^{4,5}. In the area of environmental management, modelling the rates at which industrial pollutants added to surface waters are sequestered in deep waters and purged by the river flux is critically dependent on the rate of vertical exchange³.

The results we report here were obtained on a joint Soviet-American expedition to study the physical and chemical limnology of Lake Baikal. They show that deep-water renewal is accomplished by an instability resulting from the decrease in the temperature of maximum density of fresh water with increasing pressure, and that the action of the wind or other external forcing is required to mix the surface layer downward to a depth of ~250 m before convection into the deeper layers can take place. About 12.5% of the deep-water inventory in Lake Baikal is renewed each year, corresponding to a mean deep-water residence time of 8.0 years, and the mean flux of water per unit area into the deeper layers is roughly equal for each of the lake's three deep basins. By combining the deep-water renewal rate with the observed deep-water dissolved oxygen depletion, we calculate that the mean rate of deep-water oxygen consumption by *in situ* respiration is ~4.5 μmol kg⁻¹ yr⁻¹. This consumption is balanced by a mean annual rate of photosynthetic 'new

production' in surface waters of ~27 g C m⁻² yr⁻¹. Lake Baikal is strongly oligotrophic, and nutrient elements in the surface layer are recycled on average about four times before being removed to deep waters.

Our measurements and samples were taken aboard the RV *Vereshchagin* on 15-24 July 1988, at twenty-six stations along sections crossing the three main basins of the lake (Fig. 1). Continuous vertical profile data were collected using two internally recording conductivity-temperature-depth (CTD) instruments (temperature accuracy and precision: 0.002 °C) fitted with light transmissometers. Water samples were collected at deep stations in each of the three basins using 5-litre Niskin bottles fitted with reversing thermometers. Shipboard measurements on these samples included dissolved oxygen by titration; nitrate, phosphate and silicate by colorimetry; and, as time-dependent chemical tracers, dissolved atmospheric chlorofluorocarbons (CFCs) by electron-capture gas chromatography⁶. We had planned to measure both CFC-11 and CFC-12, but unfortunately the Niskin sampling bottles were heavily contaminated with CFC-11 during shipment and we therefore report only the CFC-12 results. Samples were also collected for shore-based measurements of major ions, trace elements, tritium, helium isotopes,

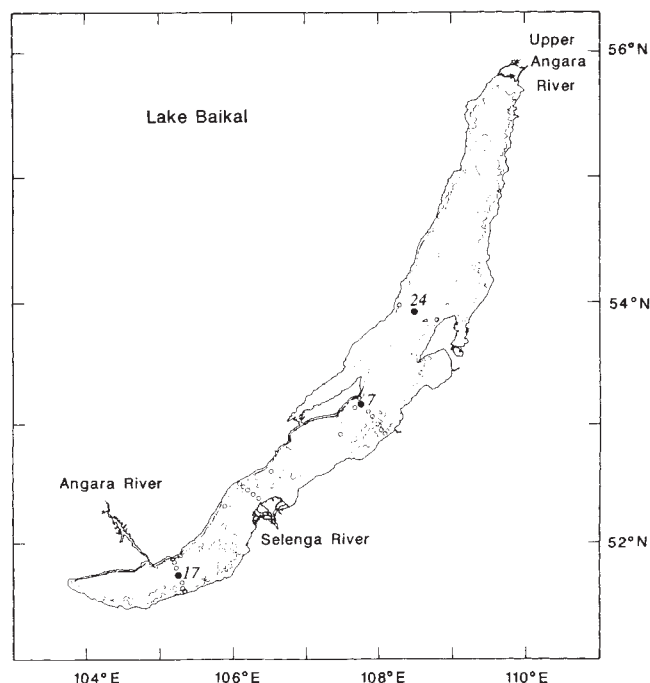


FIG. 1 Map showing the bathymetry of Lake Baikal and the locations of stations occupied by RV *Vereshchagin* during the expedition. The stations in each of the three deep basins where the water samples plotted in Figs 3 and 4 were collected are shown as filled circles and marked with station numbers, and the remaining stations are shown as open circles. Depth is contoured at 400-m intervals.

alkalinity, total inorganic carbon, carbon stable isotope ratios and suspended particulate material. The results of these measurements will be reported elsewhere.

Mechanism of deep ventilation

A commonly held belief is that deep temperate freshwater lakes like Lake Baikal, in which surface temperatures pass through the 4°C temperature of maximum density, 'turn over' twice yearly, in autumn and in spring^{7,8}. At the same time, it is well recognized that near-bottom temperatures in many deep lakes remain below 4°C throughout the year⁷⁻¹¹, and that this must in some way be related to the fact that the temperature of maximum density ($T_{\rho_{\max}}$) decreases with increasing pressure at a rate of $\sim -0.021^\circ\text{C bar}^{-1}$ (refs 12, 13). Although water near 4°C is at maximum density at the surface, it is less dense than slightly colder waters at depth, and thus cannot possibly displace by free convection any colder underlying waters that may lie closer to the $T_{\rho_{\max}}$ curve. An additional physical mechanism is therefore required to allow the displacement of colder deep water.

We propose that deep convection in such lakes takes place locally and episodically as a result of a conditional instability, the 'thermobaric' instability, which depends on the combined effects of temperature and pressure on water density^{8,14}. Consider a two-layer reversely stratified lake in the spring or autumn, when the surface layer is below 4°C and slightly colder than the deep layer (Fig. 2a). At the depth of the interface, the deep layer is nearer in temperature to the $T_{\rho_{\max}}$ curve than the surface layer, and so the interface is stable. Suppose now that the interface is moved downward by the wind, or by some other external forcing mechanism, to the depth where the interface between the two layers crosses the $T_{\rho_{\max}}$ curve and the densities of the two layers at the interface are equal (Fig. 2b). The interface is then neutrally stable. With any further deepening, or even with mixing across the interface, the overlying layer becomes denser than the underlying layer, the interface becomes unstable and deep convection ensues (Fig. 2c). Once this mechanism is triggered, the plume of convecting water will sink to the bottom unless colder (denser) waters are encountered at greater depth. This production of new deep water will tend to restore stability by decreasing the depth of the interface between the two layers, or by increasing the temperature of the surface layer through mixing with upwelled deep water. When the temperature of the surface layer equals that of the deep layer, deep ventilation must stop.

When surface layer temperatures are warmer than the deep layer but colder than the 4°C temperature of maximum density at the surface, convective waters can sink only to their local compensation depth. This will yield a temperature profile that

follows the $T_{\rho_{\max}}$ profile up to the surface, leaving a 'knee' in the temperature profile at the maximum depth at which the thermobaric instability occurred. When the surface temperatures are above 4°C, the entire upper layer is stabilized and the seasonal thermocline forms as the balance between wind mixing and buoyancy flux. Hence, seasonal convection must be considered in two parts: early spring or late autumn when the surface layers is colder than the deep layer, and deep ventilation is possible so long as there is external forcing; and late spring or early autumn when the surface layer is warmer than the deep layer, but colder than 4°C, and deep ventilation is restricted. Further, we have defined three convective regimes: the upper convective regime, which lies above the depth at which the temperature curve crosses $T_{\rho_{\max}}$; the middle regime, which can only be punctured by a conditional instability at the thermobaric compensation depth; and the lower layer, which is newer deep water that has penetrated through the middle regime by episodic deep convection.

Temperature profiles that we measured in the three deep basins of Lake Baikal show the consequences of this deep convective mechanism (see Fig. 3). At the time of our expedition, the northern and central basins had surface temperatures that were still below 4°C, whereas the southern basin had warmed above 4°C and was entering summer stratification and the associated plankton bloom. The most conspicuous feature of the temperature profiles is their division into three layers. The upper layer roughly follows the $T_{\rho_{\max}}$ curve, and the base of this layer is marked by a distinct knee in the temperature profile at ~ 250 m. The central and northern basin profiles show many temperature inversions, indicating that convection is still active in the upper layer. Local mixing driven by this convection is probably responsible for these profiles generally lying somewhat below the $T_{\rho_{\max}}$ curve. The mid-depth layer is marked by a gradual decrease in temperature from the knee downwards. Finally, the near-bottom layer of newer deep water is distinguished by an abrupt change in temperature gradient and colder temperatures several tens of metres above the bottom. Winter-time profiles measured with reversing thermometers¹¹ show temperature maxima near 200-m depth, which suggests that the summer knee is a remnant of the winter temperature structure and represents the depth to which the thermobaric instability and adjustment occurs.

We do not include the effects on density of variations in dissolved or suspended solids. The results from our Lake Baikal expedition show that variations in dissolved major ions (0.0963 ± 0.0006 salinity in parts per thousand by mass) are completely insignificant in this context (K. K. Falkner, personal communication), and that variations in particulate material below the photic zone can account for no more than $2 \times 10^{-7} \text{ g cm}^{-3}$ variations in density (W. Gardner, personal communication). Neglecting these factors is therefore valid for Lake Baikal, but their importance in other deep temperate lakes must be determined by observations.

Rate of deep-water renewal

The nature of the deep ventilation process in Lake Baikal is further revealed by our shipboard chemical measurements. Of special interest are the profiles of dissolved atmospheric CFC-12, which may be used to assess the timescale of this process^{15,16}. Surface waters of Lake Baikal that have exchanged their dissolved gases with the atmosphere and then enter the subsurface circulation, carry with them concentrations of CFC-12 that are proportional to the temporal increases of this industrially produced compound in the atmosphere. CFC-12 is extremely stable in the troposphere and in natural waters, and the history of its release and accumulation in the atmosphere is fairly well known, especially after the mid-1970s, when routine direct atmospheric measurements were undertaken¹⁷. The profiles of CFC-12 measured in the three deep basins of Lake Baikal (Fig. 3) are thus qualitatively consistent with the conclusions drawn from

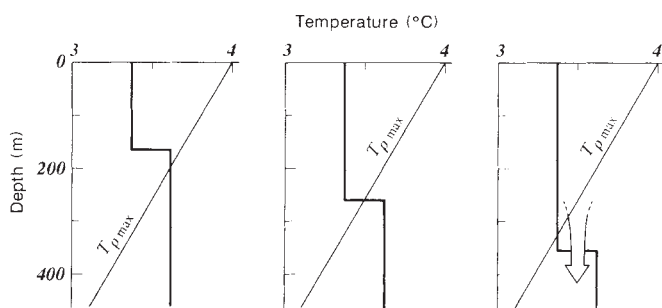


FIG. 2 Schematic representation of temperature versus depth conditions relative to the temperature of maximum density, $T_{\rho_{\max}}$, that lead to the onset of a conditional instability in freshwater temperate lakes (see text). a, Stable and reversely stratified interface at which the surface layers is further from $T_{\rho_{\max}}$ than the deep layer. b, Neutrally stable or metastable interface at which the two layers are equally close to $T_{\rho_{\max}}$. c, Unstable and convecting interface at which the surface layer is closer to $T_{\rho_{\max}}$ than the deep layer.

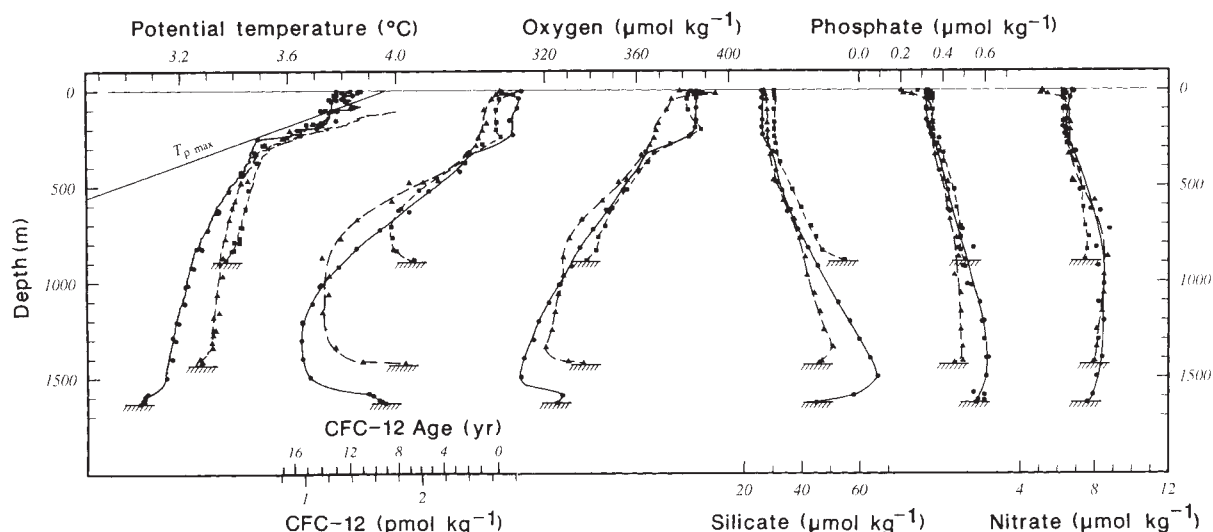


FIG. 3 Vertical profiles of temperature, CFC-12, oxygen, silicate, phosphate and nitrate in the three deep basins of Lake Baikal. Station locations are shown in Fig. 1. Values are plotted as circles and solid lines for the central basin (Sta. 7, the deepest profile); as triangles and long dashed lines for the southern basin (Sta. 17, the intermediate profile); and as squares and short dashed lines for the northern basin (Sta. 24, the shallowest profile). Temperatures are plotted as potential temperature¹³ (the temperature a water parcel would have if moved adiabatically to a pressure of one atmosphere), with the lines being the CTD measurements and the points being reversing thermometer data. Although the difference between potential

the temperature observations: the upper layer shows the highest concentrations, and hence the greatest degree of ventilation; the mid-depth layer shows decreasing concentrations and degree of ventilation with increasing depth, with a minimum in each profile located near the bottom of this layer; and the near-bottom layer shows increasing concentrations and degree of ventilation with increasing depth, indicating a higher proportion of relatively new deep water supplied by convective plumes that have penetrated through the mid-depth layer.

Our CFC-12 results show that concentrations in the upper layer, above the 250-m depth which marks the approximate limit for thermobaric instability mixing across the $T_{\rho \max}$ line, are vertically homogeneous and average $\sim 2.65 \mu\text{mol } (10^{-12} \text{ moles}) \text{ kg}^{-1}$. Although this concentration is only $\sim 82\%$ of atmospheric saturation¹⁸ (at a mean temperature of 3.76°C , a salinity of 0.096 parts per thousand, a mean barometric pressure at the lake surface of 0.947 atm, and a measured atmospheric CFC-12 dry air mole fraction of 454×10^{-12}), undersaturation is to be expected. Upwelling of deep waters displaced by deep convection mixes undersaturated waters into this layer, and seasonal stratification very near the surface impedes active exchange of underlying waters with the atmosphere, so that the observed undersaturation of $\sim 18\%$ is a dynamic response. To simplify our discussion, we assume that the 250-m upper layer supplies the deep convection, and that the CFC-12 concentration in this layer has also been 18% below atmospheric saturation in the past, even though we recognize that this latter assumption may be in error by a few per cent. We can then reconstruct, from the atmospheric growth curve and the solubility, the CFC-12 concentrations of waters which have undergone deep convection in the past. The relative ages which correspond to these source water concentrations are indicated below the CFC-12 profiles in Fig. 3.

Comparison of the source-water concentration history with the CFC-12 profiles in Fig. 3 qualitatively shows that the mean residence time of Lake Baikal deep water below 250-m depth is on the order of a decade, and that the deeper basins are ventilated more slowly, as one would expect if the rate of deep

temperature and *in situ* temperature for fresh water near the temperature of maximum density is small (a maximum in Lake Baikal of -0.025°C at 1,632 m), only the potential temperature is conserved in vertical mixing processes. The potential temperature of maximum density curve, $T_{\rho \max}$, at the Lake Baikal salinity of 0.096 parts per thousand, is shown for comparison¹³. Temperatures $>4^\circ\text{C}$ in the near-surface waters of the southern basin (Sta. 17), which extend to a surface temperature of 7.81°C , are outside the scale of this figure and are not plotted. Calculated CFC-12 relative ages for the upper layer source waters are indicated above the CFC-12 concentration scale (see text).

convection were independent of the depth of the underlying basin. However, the quantitative use of our CFC-12 results to determine ventilation rates for all the subsurface waters of Lake Baikal ultimately depends on the development of a realistic deep mixing model, including the effects of advection and eddy diffusion, which is beyond the scope of the present paper.

In the absence of such a model, deep-water renewal rates can be approximated with some simple assumptions. Because of nonlinearities in the temperature dependence of the solubility and in the rate of increase of the atmospheric source function, the time-dependent signal carried by the CFC-12 concentrations in deep waters is strictly equivalent to chronological age only if there is no mixing. Although there is surely extensive turbulent mixing associated with the production and upwelling of Lake Baikal deep waters, the temperatures in these waters cover a very narrow range and the CFC-12 atmospheric source function has been remarkably linear over the two decades preceding our expedition. The CFC-12 concentrations of waters that have entered the deep circulation from the upper layer are therefore well represented as a linear function of time over this period, with associated uncertainties of $\sim 0.05 \mu\text{mol kg}^{-1}$ or about one-half year. As a result, Lake Baikal deep-water mixtures may be regarded as having CFC-12 concentrations that are approximately characteristic of the mean ages of the various components from which they were formed.

Accordingly, the CFC-12 relative source water ages indicated in Fig. 3 may be interpreted as mean mixing ages for the individual deep-water CFC-12 observations. The least-ventilated water in Lake Baikal, found between 1,200- and 1,400-m depth in the central basin, has a mean CFC-12 mixing age of ~ 16 yr, whereas the corresponding minima in the southern and northern basins have mean CFC-12 mixing ages of ~ 14 yr and ~ 9 yr respectively. We have integrated the measured CFC-12 profiles over the volumes of each of the three basins below 250-m depth, using hypsographic curves obtained from the bathymetry in Fig. 1. The resulting mean CFC-12 concentration for the deep waters of the entire lake of $1.81 \mu\text{mol kg}^{-1}$ matches our reconstructed source function for the upper layer in early 1980. Thus, the deep

waters of Lake Baikal are ventilated by deep convection from the upper layer with a mean timescale of ~ 8.0 yr, that is $\sim 12.5\%$ is renewed each year. The equivalent mean vertical exchange flux across the 250-m isobath of the whole lake is $\sim 75 \text{ m yr}^{-1}$, and this value is about the same in each of the three basins.

To the best of our knowledge, this is the first estimate of the deep-water ventilation rate of Lake Baikal using a time-dependent chemical tracer. Previous estimates, using time-series observations of deep-water temperature and chemistry changes^{19–21}, or based on physical models of vertical transport and exchange^{21,22}, have yielded deep-water renewal rates ranging from less than 1 yr to ~ 25 yr. We believe that our estimate is considerably more reliable than this earlier work. The previous estimates using time-series observations are generally vulnerable to measurement uncertainties, which are close to the magnitudes of the observed changes, and the estimates based on physical models were only intended as rough approximations, which may be considered to agree reasonably well with our CFC-12 results.

Biological processes

The dissolved-oxygen-concentration profiles in the three basins are plotted in Fig. 3. In the upper layer, oxygen concentrations in the central and northern basins average 99% of saturation²³, whereas in the southern basin, they are slightly reduced and average 94% of saturation. These values reflect the combined effects of atmospheric exchange, photosynthetic production in a photic zone that extends well into this layer, even during the development of a near-surface seasonal thermocline, and respirative consumption. The somewhat lower oxygen saturation in the upper layer of the southern basin may result from the onset of the plankton bloom in this basin, which reduces water transparency and restricts oxygen production to shallower depths while increasing the role of respirative recycling in the remainder of the upper layer.

In the deep waters of Lake Baikal, the dissolved-oxygen profiles show a similar structure to the CFC-12 profiles. Qualitatively, one would expect such a correlation because oxygen removal by *in situ* respiration should be most pronounced in waters that are most isolated from renewal by vertical mixing. This relationship is shown more clearly by plotting oxygen concentration against CFC-12 concentration (Fig. 4). The three basins show remarkably similar and nearly linear trends in the

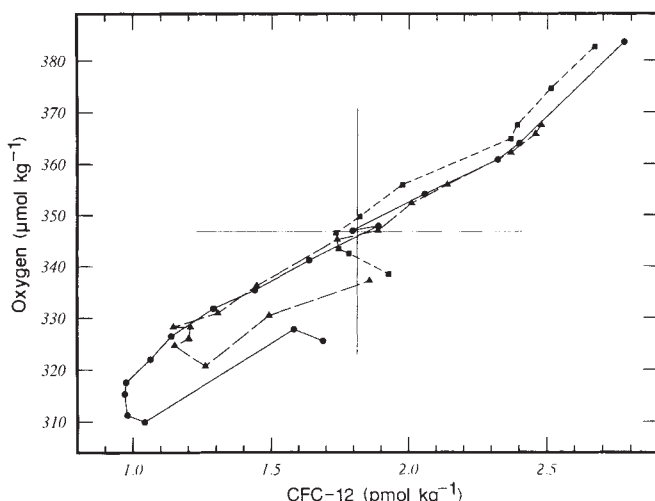


FIG. 4 Oxygen concentration against CFC-12 concentration for waters at depths of >225 m in the three deep basins. Stations and symbols are the same as in Fig. 3. Crossed horizontal and vertical lines mark the mean oxygen and CFC-12 concentrations for all waters of the lake below a depth of 250 m.

mid-depth layer above the deep concentration minimum. The trends in the near-bottom layer, below the concentration minimum, show similar slopes displaced toward lower oxygen concentration. This displacement may be the result of respiration of organic detritus at the sediment/water interface added to the effects of oxidation in the water column.

Where the rate of *in situ* oxygen consumption in the deep waters is zero-order (namely, independent of concentration), and therefore a linear function of time, and the CFC-12 concentration is also a linear function of time as discussed above, then the relationship between oxygen concentration and CFC-12 concentration resulting from deep mixing will also be linear, and the slope of this relationship will be proportional to the *in situ* oxygen-consumption rate. Accordingly, the mean slope of the mid-depth layer measurements in Fig. 4 is equivalent to an *in situ* oxygen-consumption rate of $3.8 \mu\text{mol kg}^{-1} \text{ yr}^{-1}$. To calculate the mean oxygen-consumption rate of the combined mid-depth and near-bottom layers, we have carried out a hypsographic integration of the oxygen profiles in each of the three basins below a depth of 250 m. The resulting mean oxygen concentration for the deep waters of the entire lake is $347 \mu\text{mol kg}^{-1}$. Assuming a source-water oxygen concentration of $383 \mu\text{mol kg}^{-1}$ in the upper layer, and a mean ventilation time of 8.0 yr yields a mean oxygen-consumption rate for the entire deep-water regime of $4.5 \mu\text{mol kg}^{-1} \text{ yr}^{-1}$.

Nutrient profiles vary roughly inversely to those of oxygen (Fig. 3), showing maxima near the base of the mid-depth layer. The deep-water increases in nitrate and phosphate relative to the upper layer are due to the *in situ* oxidation of sinking particulate organic material, primarily diatoms¹⁹; the corresponding increases in silicate are due to the dissolution of diatom frustules and other biogenic opal¹⁹. Nutrient decreases in the near-bottom layer again reflect the larger contribution to this layer of relatively new deep water of upper-layer origin, as discussed above. Whether nitrogen or phosphorus limits production in surface waters depends on the ratio in which these elements are made available to phytoplankton. In Lake Baikal, the mean slope of nitrate versus phosphate in molar units is ~ 9 . In freshwater lakes, molar slopes of ~ 22 or less generally indicate that nitrogen limits growth, and this is probably the case in Lake Baikal, although slopes as low as 11 may be phosphorus-limited if there is atmospheric nitrogen fixation in surface waters²⁴.

Photosynthetic primary production in Lake Baikal may be considered as the sum of 'new production' resulting from the input of nutrients to the upper layer from below, and 'regenerated production' resulting from nutrient recycling in the upper layer²⁵. In this context, the riverine input of nutrients to the upper layer is insignificant²⁶. The mean annual primary productivity of Lake Baikal, based on oxygen and radiocarbon bottle incubation experiments, is $\sim 120 \text{ g C m}^{-2} \text{ yr}^{-1}$ (ref. 26). From our determination of the mean oxygen-consumption rate in deep waters, plus an estimate of the flux of organic carbon sequestered in sediments, it is possible to determine the mean flux of organic carbon from the upper layer into the deep waters. This flux is the portion of the primary production that is not recycled, and hence is equal to the new production. Assuming a mean mole ratio of carbon dioxide regeneration/oxygen consumption during respiration of 0.8, our mean deep-water oxygen-consumption rate of $4.5 \mu\text{mol kg}^{-1} \text{ yr}^{-1}$ corresponds to a mean new production flux across the 250-m isobath of $26 \text{ g C m}^{-2} \text{ yr}^{-1}$. To this must be added a flux of $\sim 1 \text{ g C m}^{-2} \text{ yr}^{-1}$ for organic carbon sequestered in deep sediments, and not subject to oxidation through diagenesis and methane production (D. Edgington, personal communication). Thus, our oxygen and CFC-12 results yield a mean annual rate of new production for Lake Baikal of $27 \text{ g C m}^{-2} \text{ yr}^{-1}$.

The productivity of Lake Baikal is thus shown to be strongly oligotrophic and approaching typical open-ocean values²⁵. Using the values given above, the ratio of new production to

primary production, f , is ~ 0.2 in Lake Baikal, and so also approaches open-ocean values²⁵. As f is the probability that a nutrient element will be lost from the upper layer and not

recycled, our results show that nutrients in the upper layer are recycled an average of about four times before they are lost to deep waters. □

Received 9 October; accepted 31 December 1990.

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ACKNOWLEDGEMENTS. This research could not have been carried out without the collaboration of many scientists, technicians and administrators in the Soviet Union, the United States and Canada. We are especially grateful to N. Koval, Y. Kusner, C. Lange, V. Nikolashin, P. Salameh, F. Van Woy, A. Vershinsky, N. Williams, R. Williams, and the officers and crew of the RV *Vereshchagin*. Ship time and local logistic support were provided by the Limnological Institute at Irkutsk and Listvianka. This work was organized under the auspices of Working Group VIII of the US-USSR Bilateral Agreement on Environmental Protection, with financial support from the US NSF, the USSR Goskomgidromet, the Siberian Branch of the USSR Academy of Sciences, and the Government of Canada-Fisheries and Oceans.

Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments

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Traffic of MHC molecules dictates the source of peptides that are presented to T cells. The intracellular distribution of MHC class I and class II molecules reflects the dichotomy in presentation of antigen from endogenous and exogenous origin, respectively. In human B lymphoblastoid cells, class I molecules are present in compartments constituting the biosynthetic pathway, whereas class II molecules enter structures related to lysosomes during their biosynthesis.

T CELLS recognize short peptides from processed antigens in the framework of major histocompatibility complex (MHC) class I or class II products¹. Class I molecules consist of a membrane glycoprotein heavy chain that contains all peptide-binding features, in association with the light chain, β_2 -microglobulin². With few exceptions, class I heavy chains seem to need β_2 -microglobulin and a tightly bound peptide for their conformational integrity^{3–5}. Class I molecules take up peptides derived largely from proteins synthesized by the antigen presenting cell (APC) itself, early in the course of biosynthesis, presumably in the endoplasmic reticulum (ER)^{3,4}. The present view is that MHC class I molecules follow the constitutive secretory pathway from ER through Golgi to the cell surface^{6,7}.

Class II molecules consist of two MHC-encoded membrane glycoproteins chains, α and β . The class II $\alpha\beta$ heterodimer assembles transiently in the ER with a third chain, the invariant chain, to form a heterotrimeric complex⁸. The invariant chain

could serve to prohibit or modify binding of endogenous peptide to class II molecules in the ER. It might also function as part of a targeting signal directing class II molecules to the endocytic route^{9,10}. Class II molecules preferentially bind peptides derived from exogenous antigens degraded in the endocytic pathway¹¹, although the endogenous pathway may also be operative^{12,13}. The antigen-presenting capacity of cells bearing class II shows variation according to cell type¹⁴, and is likely to be related to the proteolytic machinery and intracellular routes followed by antigen and class II molecules. Understanding antigen presentation requires a detailed analysis of these processes in different types of APC. Human B-lymphoblastoid cells transformed by Epstein-Barr virus (EBV) are widely used as APC and can present a variety of soluble antigens^{14,15}. Early endosomes were recently identified in such B cells as the initial site where class II molecules meet antigen¹⁶. Here we arrive at a different conclusion.

We show that in the course of biosynthesis, class II molecules are routed to compartments related to lysosomes. In the Golgi region we observe elements that are positive for class I molecules; these are presumed to be part of the constitutive secretory pathway. We also observe structures positive for cation-dependent and cation-independent mannose 6-phosphate receptors (CD-MPR, CI-MPR) in association with the morphologically defined *trans*-Golgi reticulum (TGR)¹⁷. Such structures have been reported to transport MPR-lysosomal enzyme complexes to late endosomes^{18,19}. Finally, we identified elements near the Golgi that are strongly labelled for class II molecules but were negative for class I and MPR. These elements are probably part of the TGR, but in the absence of an immunocytochemical marker that uniquely identifies the TGR this

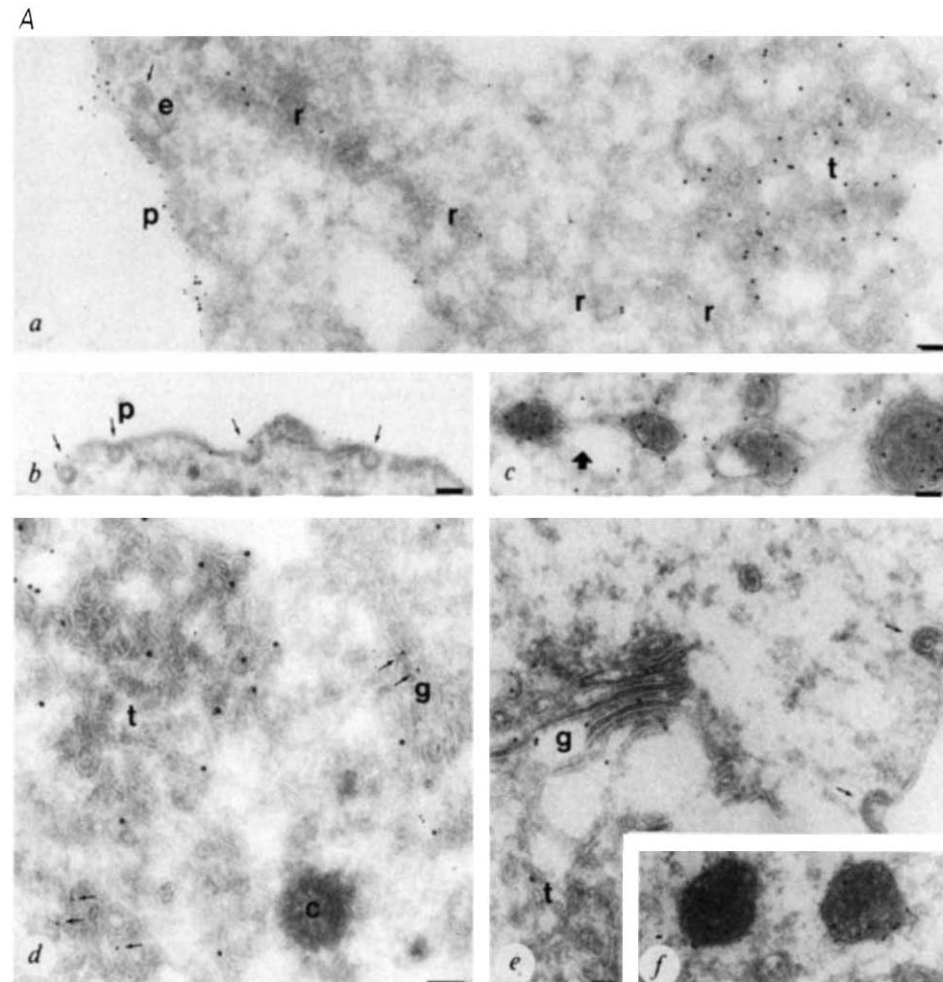
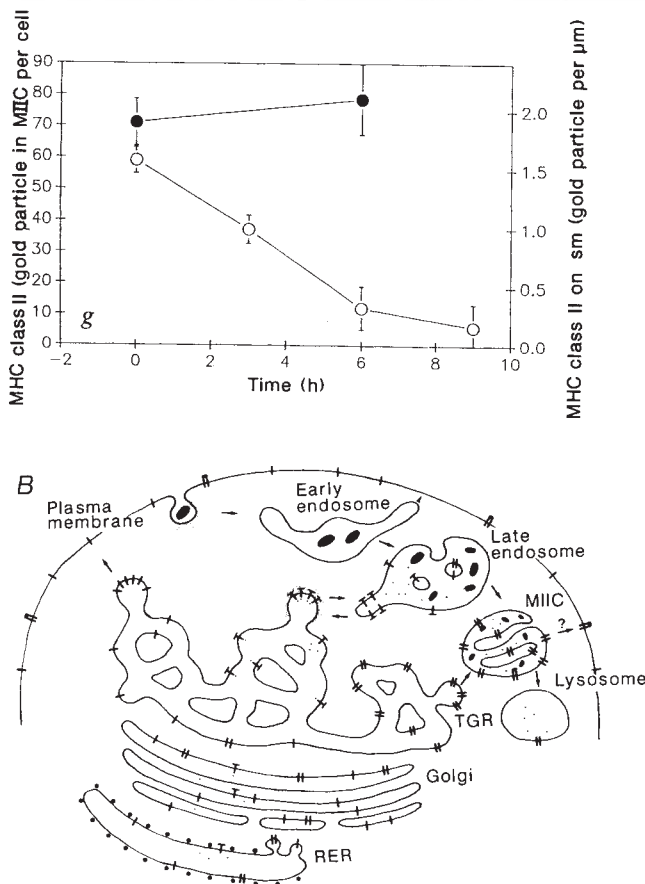


FIG. 1 A, Distribution of MHC class I and II molecules and invariant chain. Double labelling of class I (gold: 10 nm) and II (gold: 15 nm) molecules (a–d, f). a, MHC class I and II molecules are localized at the plasma membrane (p) and in the RER (r). The endosome (e) is devoid of label (the arrow points at a coated bud on the endosome). Note that the TGR domain (t) contains class II molecules only. b, Plasma membrane (p) containing coated pits (arrows) are devoid of label. c, Characteristic image of MIIC. They are devoid of label for class I molecules (see also Figs 2, 3a, 4b, c, d, 5a, b, c). d, TGR (t)/Golgi (g) area showing labelling of both class I and II molecules in the Golgi, but segregation of class I (arrows) and II molecules in the TGR. c, centriole. e, Single labelling of invariant chain. Labelling is restricted to the ER (not shown) and Golgi complex (g). Some labelling was observed in the TGR region (t). Note that the plasma membrane and coated pits (arrows) are devoid of label. f, Visualization of class I and II molecules in endocytotic multivesicular bodies. These structures could only be visualized in cells cultured in the presence of protease inhibitors and probably represent an intermediate stage in the breakdown of cell-surface molecules. Bars represent 100 nm. g, The effect of cycloheximide (CHX) treatment on class II labelling in MIIC. To study whether MHC class II molecules enter the MIIC through the biosynthetic route, cells were cultured in the presence of CHX ($100 \mu\text{g ml}^{-1}$) for periods indicated in the figure. For

each time-point, the number of class II gold particles in MIIC was determined in 300 cells. There is a gradual decrease of class II labelling in the MIIC (open circles). Labelling density of plasma membrane remained constant for at least 6 h (closed circles). Thus class II molecules are delivered biosynthetically to the MIIC. B, Traffic of MHC molecules. The pathways travelled by MHC molecules are summarized diagrammatically: I, MHC class I; II, MHC class II, and T, MPR; square dots nearby represent MPR ligand. Antigen, as it is progressively degraded, is visualized as a filled ellipse decreasing in size.

METHODS. The human EBV-transformed B cell line JY⁷ was maintained in suspension in DMEM supplemented with 10% (v/v) fetal calf serum (FCS). In some experiments (f) cells were incubated overnight with a mixture of aprotinin ($5 \mu\text{g ml}^{-1}$), trypsin inhibitor ($5 \mu\text{g ml}^{-1}$) and leupeptin ($10 \mu\text{g ml}^{-1}$). Samples were fixed in either 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, for 2 h at 37 °C (Figs 1, 2, 4 and 5) or in 1% acrolein and 2% formaldehyde in the same buffer for 2 h at 37 °C and then for 2 h at 4 °C (Fig. 3) and processed for cryoimmunoelectron microscopy as described by Slot *et al.*⁴⁷. Briefly, cryosections ($\sim 100 \text{ nm}$) were incubated at room temperature with antibodies (usually dilutions of 1:500, anti-DNP was used at 1:600 or 1:9,000, see Fig. 5) for 30–60 min and with protein A/gold complexes⁴⁸ for 20 min, respectively. In the case of double or triple labelling, 0.5% glutaraldehyde for 5 min was used after the gold labelling to prevent possible colabelling⁵¹. At optimal antibody dilutions no background labelling over nuclei and mitochondria was seen. Irrelevant control antibodies (rabbit anti-human granzyme B) did not give any labelling. The electron microscopic observations were made with a JEOL 1200 EX.



remains unproven. Further, we identified a compartment with lysosomal characteristics that is enriched in class II molecules, and is devoid of class I and both CI- and CD-MPR. We refer to this structure as MHC class II compartments (MIIC).

The MIIC are mildly acidic and contain the lysosomal enzyme β -hexosaminidase as well as the typical lysosome-associated membrane proteins lamp-1 (ref. 21) and CD63 (ref. 22). Timed addition of an endocytosis marker shows the appearance of the marker in MIIC between 30 and 60 min. MIIC are not labelled for CD-MPR and CI-MPR, which are characteristic for late endosomes^{18,19,23}, classifying the MIIC as close relatives of lysosomes.

Biosynthetic pathway of MHC molecules

In the EBV-transformed human B-lymphoblastoid cell line JY, the biosynthetic route of class II molecules (in contrast to that of class I molecules) intersects the endocytotic pathway after passage through the Golgi but before their arrival at the cell surface⁷, favouring the idea that during their biosynthesis, class II molecules meet and bind exogenously derived antigenic peptides in an endocytotic compartment^{10,24,25}. JY cells possess compartments that contain abundant class II molecules⁷. We therefore characterized the steady-state distribution of class I and II molecules using the immunogold technique on ultrathin cryosections. The results of this analysis are summarized in

Table 1 and Fig. 1B. Representative immunolabelling images of the steady-state localization of class I, class II and invariant chain in JY cells are shown in Fig. 1A. Invariant chain was largely confined to the ER and Golgi complex (Fig. 1Ae). The TGR in these cells (as for other cell types¹⁷) consists of reticular cisternae, with budding profiles and coated vesicles. The TGR in JY cells is located at the *trans* face of the Golgi complex. The distance of TGR from Golgi is usually greater than that between the individual cisternae of the Golgi (see schematic representation, Fig. 1B). Further, the TGR is often seen to project laterally from the Golgi. Examples of TGR are shown in Figs. 1Ad, 2c, 5b, indicated as 't'. Small electron-lucent structures of ~80–100 nm, in close association with the TGR, were labelled for either class II or, but to a lesser extent, MHC class I, but not for both. They did not label for galactosyltransferase (data not shown), a resident protein in the *trans*-Golgi cisternae²⁶, indicating that these profiles do not belong to the *trans* cisternae of the Golgi complex. Our observations suggest that class I and II molecules are sorted in TGR membranes for transport to the plasma membrane and endocytotic pathway respectively, in agreement with the important role of TGR in protein sorting¹⁷. We suggest that the class II-positive profiles seen in Figs 1Aa and d, 2c, 5b are involved in delivery of MHC class II molecules to MIIC. These profiles disappear after 2 h of cycloheximide (CHX) treatment (see below).

TABLE 1 Summary and semi-quantitative analysis of immunoelectron microscopy data

(a) Distribution of marker molecules								
	RER	Golgi	TGR	MIIC	Lysosome	Late endosomal	Early endosomal	Plasma membrane
MHC class II	+/-	+	++	+++	-	+/-	-*	++
Invariant chain	+++	++	+	-	-	-	-	-
MHC class I	+	++	+	-	-	+/-	-*	+++
Galactosyltransferase	-	+	+/-	-	-	-	-	-
CI-MPR	+/-	+	+	-	-	++	-	-
CD-MPR	-	+	+	-	-	+	-	-
cath. D/ β -glu/lap	-	-	-	-	+	+	-	-
β -hexosaminidase	+++	++	+	+	+	+	-	-
h-lamp 1	-	-	-	++	++	+	-	-
CD 63	-	-	-	++	++	+	-	-
DAMP	+/-	+/-	+	+	++	++	+/-	-
Internalization in min at 37 °C								
Transf. 60 min			-	-	-	-	+	+
BSA 60 min			-	-	-	+/-	+	+/-
HRP 60 min			-	++	+	+	+	+/-
CF 10			-	-	-	-	+	+
CF 10 + 20 min chase			-	-	-	+	+	+
CF 10 + 50 min chase			-	+	+	+	+	+
(b) Gold particles per 5 μm^2 (for each compartment)								
	RER	Golgi	TGR§	MIIC	Endosomes			
MHC class II	4	37	97§	403	8			
MHC class I	17	158	48§	4	17			
CI-MPR	4	55	36§		149			

Distribution of marker molecules established by counting gold-particle labelling for a particular antigen in a certain compartment and comparing the intensity of label to the other antigens. b, Semi-quantitative distribution of the membrane proteins class I, class II and CI-MPR over the intracellular parts, which are of particular relevance for this study. Owing to possible differences in labelling efficiency among different organelles using cryosections⁴⁰, these data have only comparative value.

* <5% where weakly labelled for MHC class I and II together.

† Only in the *trans*-Golgi cisternae.

‡ Continuous internalization is observed because CF adheres to the plasma membranes.

§ No co-localization for any of the three markers quantitated within the same profile.

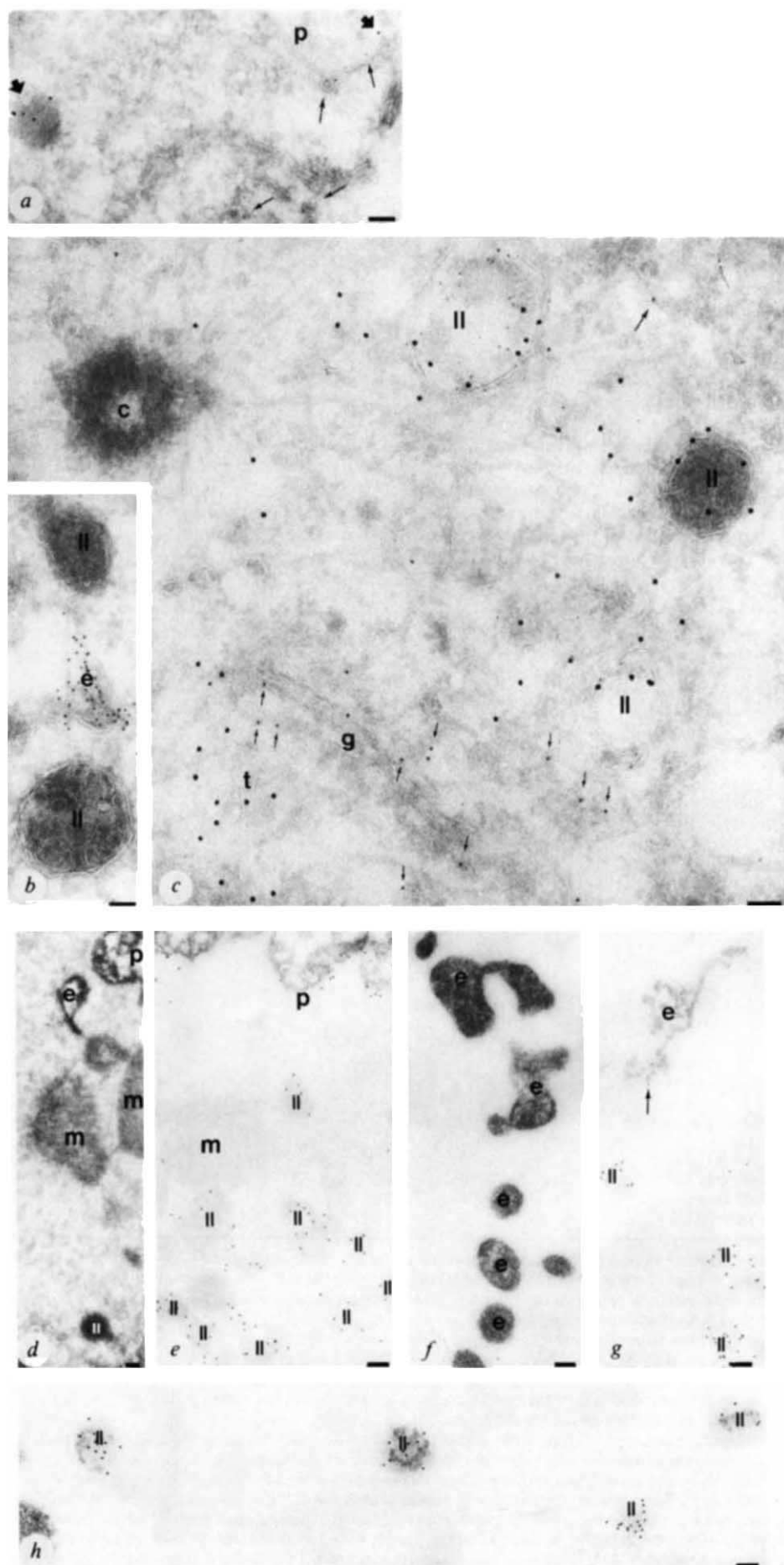
|| No label detected above background levels.

Nonspecific background labelling (three gold particles per 5 μm^2) is subtracted. About 2,000 gold particles counted in 30 cells (micrographs from medial cell profile). Endosomes are defined as HRP and CI-MPR-positive structures.

Rabbit antibodies against the following protein markers were used: MHC class II⁷; invariant (C-terminal, τ CGGH) chain⁴⁹ from B. Dobberstein; MHC class I heavy chain⁵⁰; galactosyltransferase from E. Berger; CI-MPR and CD-MPR, cathepsin D (cath. D), hexosaminidase, lysosomal acid phosphatase (lap) from K. Von Figura; β -glucocerebrosidase (β -glu) from J. Tager; lamp-1 from M. Fukuda; monoclonal antibody RUU 228 recognizing human CD63 from J. Sixma²²; DAMP (anti-DNP) (DAKOPATTS); human transferrin (Transf.) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam; bovine serum albumin (BSA) (Biomakor); horseradish peroxidase from G. Griffiths. Cationized ferritin (CF) was from Miles.

FIG. 2 Positioning of the MIIC in the endocytotic route. *a*, Double labelling of human transferrin (gold: 10 nm) and class II molecules (gold: 15 nm). Transferrin gold (thin arrows) is observed at the plasma membrane (p), in coated pits and in small endocytotic vesicles. Class II molecules (thick arrows) are present at the plasma membrane and in the MIIC at the left. Note the absence of transferrin from the MIIC. *b*, Single labelling of BSA with 10-nm gold. The endosome (e) is strongly labelled for BSA, whereas the MIIC (II) are almost negative. *c*, Triple labelling of HRP (gold: 5 nm), class I (gold: 10 nm) and class II (gold: 15 nm). Cells were allowed to take up HRP (10 mg ml^{-1}) for 1 h, followed by fixation. Class I molecules (arrows) are present in the Golgi complex (g) and TGR (t), class II labelling is seen in TGR and MIIC (II). Note the segregation of class I and II molecules in the TGR region. MIIC (II) do not contain any class I gold. Note that the most electron-dense MIIC is devoid of HRP labelling. MIIC were often observed near centrioli (c). TGR including class II domain did not label for HRP. *d-h*, Internalization of cationized ferritin (CF). JY cells were incubated with CF ($1:500$) for 10 min at 37°C and recultured for 0 (*d, e*), 20 (*f, g*) and 50 min (*h*) in the absence of CF. Because CF is itself electron-dense, sections *e, f, g* and *h* were not contrasted with uranyl acetate. Class II molecules were labelled with 15-nm gold. *d, e*, After 0-min reculture, CF (flocculent density) is observed at the plasma membrane (p) and in the endosome (e). CF is absent from the MIIC (II) which show class II gold (*d, e*). *m*, Mitochondria. *f, g*, After 20-min reculture, CF is observed in endosomes (e). MIIC are devoid of CF. There is one class II gold particle (arrow) in an endosome. *h*, After 50-min reculture, CF has reached the MIIC (II). Note that CF is present in three of the four MIIC shown. Bars, 100 nm.

METHODS. Cryoimmunogold labelling was as described in the legend to Fig. 1. *a*, JY cells were incubated with human transferrin ($50 \mu\text{g ml}^{-1}$) at 37°C for 60 min, followed by fixation in glutaraldehyde. *b*, JY cells were incubated in 100% FCS for 1 h.



At the plasma membrane, class I and II molecules but not invariant chain were detectable, in agreement with earlier biochemical observations⁸. Coated pits were largely devoid of class I and II molecules (Fig. 1*Ab*; >95% coated pits out of 1,000 counted were negative), in accordance with the limited rate of spontaneous internalization in these cells^{7,27}. This agrees with experiments in which class II molecules in coated pits could not be detected in cells labelled at 0 °C with antibody-gold conjugate, as opposed to the situation after 1 min at 37 °C (ref. 28). This, too, is an argument against an important role for coated pits as a principal site for spontaneous internalization of MHC molecules. Only when cells were exposed to the protease inhibitors leupeptin, aprotinin and antitrypsin, were class I and II molecules frequently observed in endocytotic multivesicular bodies (MVBs) (Fig. 1*Af*), suggesting that in the absence of protease inhibitors these molecules would have been degraded. These MVBs also dramatically increase in number when the cells are incubated overnight with the protease inhibitors, and probably represent an intermediate station in the degradative route of MHC molecules. In concordance with this suggestion, the ratio of class I to class II molecules was similar in these MVBs and at the cell surface (quantitative data not shown). The MIIC changed neither in number nor in labelling intensity for class II molecules on exposure of cells to protease inhibitors.

The MIIC were located mainly in the cell centre, contained arrays of internal membranes and had a diameter of 200 to 300 nm (Figs 1*Ac*, 2*a-e*, *g*, *h*, 3*a*, 4*b-d*, 5*a-c*). Each medial cell profile contained about seven MIIC.

To show that class II molecules enter MIIC by the biosynthetic route, protein synthesis was blocked for different periods with

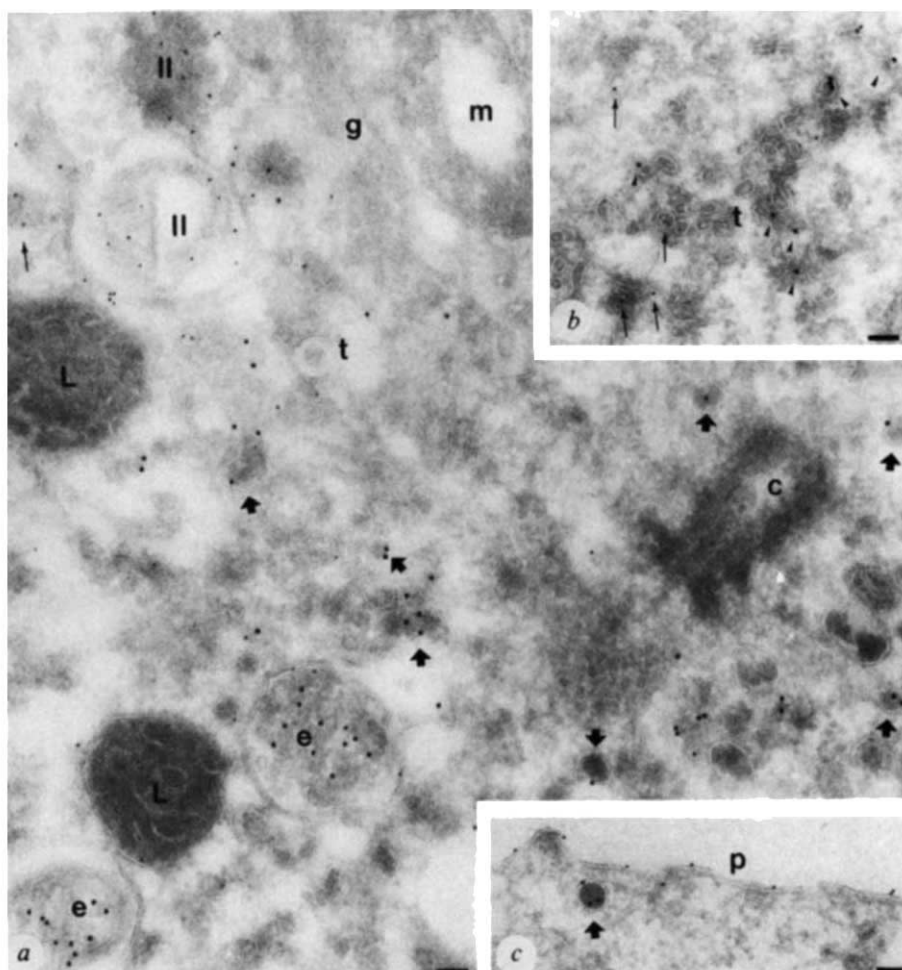
CHX²⁹, followed by fixation and immunoelectron microscopy. Quantitative electron microscopy revealed that the class II labelling in the MIIC rapidly decreased with time after addition of CHX, in agreement with the biosynthetic, as opposed to endocytotic, origin of the class II molecules in these compartments. The intensity of class II labelling at the cell surface did not greatly decrease as a consequence of CHX treatment (Fig. 1*Ag*). Class II molecules did not completely disappear from the few remaining MIIC after prolonged incubation (up to 9 h) with CHX, presumably because intracellular traffic is somewhat impaired in CHX-treated cells as analysed biochemically (data not shown). Internalization and recycling of transferrin receptor as well as the formation of coated pits³⁰ were not affected in cells exposed to CHX for up to 8 h (electron microscope data not shown, but available as Supplementary Information). Exposure of JY cells to brefeldin A (which inhibits exit of proteins from the ER³¹) gave similar results with respect to MIIC to those obtained after CHX treatment (not shown).

We conclude that in JY cells, class II molecules enter the MIIC largely by the biosynthetic route, and not by endocytosis of surface class II molecules.

Class II compartments part of endocytotic route

Internalization of exogenous antigen is followed by proteolytic processing and binding of resulting peptides to class II molecules, at an as yet unidentified position in the endocytotic route^{9-11,32}. First, a more precise definition is required in view of the subdivision of the endocytotic route (coated pits, coated vesicles, early endosomes, late endosomes and lysosomes), each identifiable and with specialized functions^{33,34}. Second, positioning of class II molecules in the endocytotic pathway

FIG. 3 Segregation of class II molecules and CI-MPR. Double labelling of the late endocytotic marker CI-MPR (gold: 15 nm) and class II molecules (gold: 10 nm). *a*, MIIC (II) do not show CI-MPR label and late endosomes (e) do not or hardly contain class II label. The lysosomes (L) are MPR-negative and show a few particles for class II. The figure shows many electron-dense MPR-enriched vesicles (large arrows) and a class II-containing vesicle (small arrow). *b*, Segregation of class II and CI-MPR in the TGR. Arrowheads denote CI-MPR and the arrows class II molecules in the TGR area. No vesicles were observed containing both CI-MPR and class II molecules. *c*, Electron-dense CI-MPR-containing vesicle (arrow) at the cell periphery. These characteristic vesicles on no occasion contained class II molecules. Note that the plasma membrane (p) is not labelled for CI-MPR. METHODS. Cryoimmunogold labelling on paraformaldehyde/acrolein-fixed cells was as described in the legend to Fig. 1. Bars, 100 nm.



deserves close scrutiny because of possible cell type-specific differences in class II distribution that might correlate with the antigen-presenting capacity of the APC.

Transferrin as well as transferrin/horseradish peroxidase (HRP) conjugates³⁵, a receptor ligand that recycles predominantly but not exclusively through early endosomes³⁶, were analysed to identify the early phase of the endocytotic route. Transferrin and transferrin-HRP added for 30 min were localized at the cell surface, in coated pits (Fig. 2a), in early endosomes and in tubular structures (not shown) morphologically³⁷ distinct from MIIC. The uptake of two fluid-phase endocytotic markers (BSA, which is rapidly degraded and HRP, which is more slowly degraded³⁸), added for 1 h at 37 °C, allowed labelling of the degradative endocytotic route. BSA was present in endosomes but only very rarely (<3%) detected in MIIC (Fig. 2b). HRP was found in both endosomes and in ~50% of the MIIC (Fig. 2c). Golgi and TGR domains, including the class II profiles, did not label for BSA or HRP (Fig. 2c). The difference in staining between HRP and BSA may be explained by the more rapid degradation of BSA³⁸. We next performed a kinetic experiment with a nondegradable marker molecule, cationized ferritin (CF)³⁴. Uptake of CF was allowed for 10 min, and cells were incubated for different periods in the absence of CF in the medium. Ultrathin cryosections were then labelled for class II. After a 10-min pulse, only peripheral early endosomes were occupied by CF (Fig. 2d, e), whereas after a 20-min incubation, CF was found also in endocytotic vacuoles (Fig. 2f) and tubules (Fig. 2g) deeper in the cells. These structures displayed very little label for class II molecules. After a 50-min incubation, ~30% of the MIIC had been reached by the tracer (Fig. 2h). We conclude that MIIC are located late in the endocytotic route.

Class II compartments and lysosomes

The above observations indicate that MIIC are either endosomes or lysosomes, both being part of the endocytotic pathway. To distinguish further between these possibilities, we performed double and triple labelling experiments with anti-class II antibodies and antibodies specific for markers of the different endocytotic compartments. Colocalization of class II molecules in MIIC with markers of early endosomes was not observed

(Fig. 2a). The late endocytotic nature of MIIC was then investigated. Both CD-MPR and CI-MPR, markers for late endosomes^{18,19,23}, were sparsely present throughout the ER, Golgi and TGR, whereas abundant labelling was observed in tubulovacuolar profiles of endosomes (CI-MPR is shown in Fig. 3). Of interest, TGR showed no colocalization of MPR and class II molecules in individual profiles (Fig. 3b, Table 1b). Note that the electron-dense MPR vesicles (Fig. 3a) observed in the TGR area and in the cell periphery (Fig. 3c) are distinct from the electron-lucent vesicles (80–100 nm) containing class II molecules (Figs 1Aa, d, 2c, 3a and 5b). These MPR-positive vesicles may be transport vesicles of lysosomal enzymes from TGR to late endosomes^{18,20}. Anti-CD-MPR and -CI-MPR did not label the MIIC which are therefore likely to be different from late endosomes. Both MPR and class II were almost absent from dense classical lysosomes (Fig. 3a).

We next analysed the localization of the resident lysosomal membrane proteins lamp-1 (ref. 21) and CD63 (ref. 22). Structures positive for lamp-1 and CD63, but devoid of class II molecules were endosomes (Fig. 4a) and dense lysosomes^{21,22}. Positive staining for lamp-1 and CD63 with class II molecules was found for MIIC (Fig. 4b, c). The lysosomal enzyme β -hexosaminidase, prominent in JY cells, was also present in MIIC (Fig. 4d). Combined with the lack of MPR, this positions the MIIC late in the endocytotic route, not as late endosomes but as close relatives of lysosomes.

We then treated the cells with the weak base 3-(2,4-dinitroanilino)-3-amino-N-methyldipropylamine (DAMP) to identify by immunoelectron microscopy the accumulation of this base in acidic structures³⁹. The intensity of labelling differed among the different compartments (Fig. 5 and Table 1): early endosomes labelled weakly; TGR and MIIC, moderately; and late endosomes and lysosomes were strongly positive. DAMP labelling in cryosections cannot be used to establish exact pH values, because of differences in the subcellular matrix⁴⁰ that may affect the penetration of DAMP or access to the anti-DNP antibody. One of the most acidic sites in the cell, the intermembrane space of mitochondria⁴¹, was labelled most intensely (Fig. 5d).

Addition of chloroquine, a weak base often used as an

FIG. 4 MIIC are related to lysosomes. Double labelling of class II molecules (gold: 15 nm) with different late endocytotic and lysosomal markers (gold: 10 nm). a, Double labelling of lamp-1 and class II molecules. Endosomes (e) are labelled for lamp-1, but not for class II molecules, whereas the plasma membrane (p) is solely labelled for class II molecules. m, Mitochondria. b, As a, MIIC (II) are labelled for lamp-1 and class II molecules whereas the plasma membrane (p) is only labelled for class II molecules. c, Double labelling of CD63 and MHC class II molecules. MIIC contain both CD63 and class II molecules. d, Double labelling of β -hexosaminidase and class II molecules. Both molecules colocalize to the MIIC (II).

METHODS. Cryoimmunogold labelling was as described in the legend to Fig. 1. Bars, 100 nm.

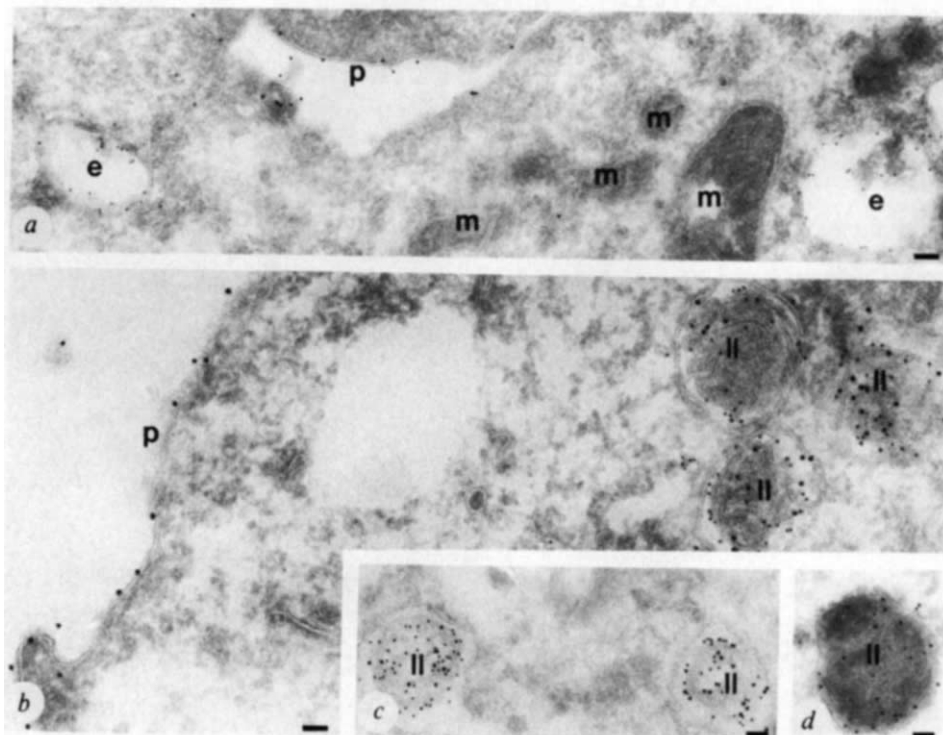
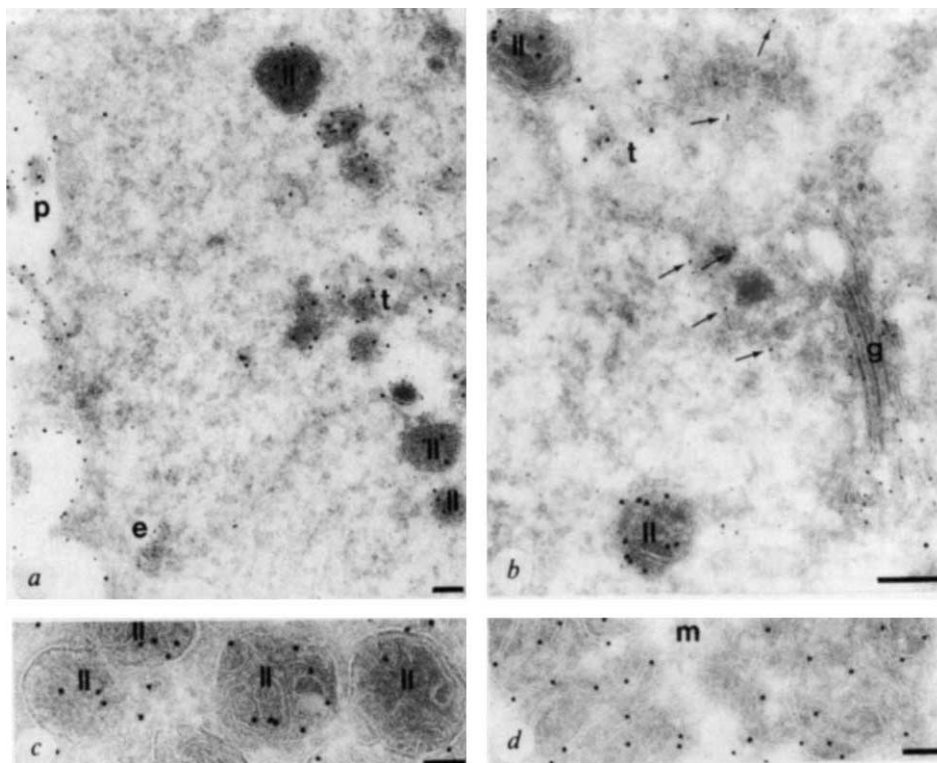


FIG. 5 MIIC compartments are acidic. *a, b*, Triple labelling for DAMP (gold: 5 nm), class I (gold: 10 nm) and class II (gold: 15 nm). Anti-DNP staining at a dilution of 1:9,000. *a, b*, DAMP labelling is concentrated in the endosome (e) but is also observed in TGR(t) and MIIC. Note that the endosome is not labelled for MHC molecules. *b*, TGR(t) subdomains enriched in class I (arrows) or class II molecules can be seen in *b*. MIIC (II) do not show any class I labelling. Class I and II molecules are both present at the plasma membrane (p) and the Golgi cisternae (g). *c, d*, Single labelling of DAMP (gold: 15 nm). Anti-DNP staining at a dilution of 1:600. DAMP accumulation is shown in MIIC (II) (*c*) and in the inter-membrane space of mitochondria (m) (*d*). Bars, 100 nm.

METHODS. Cryoimmunogold labelling was as described in the legend to Fig. 1. Note that the DAMP molecules pass membranes and are therefore not restricted to the vacuolar system.



inhibitor in immunological assays^{8,10,11,14}, demolished the microarchitecture of the endocytotic pathway, including the MIIC (not shown), and may explain the inhibitory effects of lysosomotropic agents on class II-restricted antigen presentation. We conclude that MIIC are a subpopulation of lysosomes.

Discussion

We have characterized the compartment in which newly synthesized class II molecules enter the endocytotic route in EBV-transformed B cells. We observe separate distributions of class I and class II molecules in Golgi-associated electron-lucent structures. The class II-positive profiles (Fig. 2c) could not be labelled with endocytotic tracers and may be involved in the transport of class II molecules to the MIIC. Exogenously added molecules can reach these MIIC, but only after traversing most of the endocytotic route. All proteases located in the endocytotic pathway and in the MIIC could thus participate in the generation of peptides for presentation. Double and triple labelling with early and late endocytotic and lysosomal markers revealed that the MIIC are related to lysosomes. These results emphasize an important aspect of lysosomal function. Rather than these structures being only the end-station of a variety of catabolic routes, proteins may exit from them and participate in processes such as antigen presentation.

Our findings are in contrast to the observations of Guagliardi *et al.*¹⁶, who reported colocalization of class II molecules, invariant chain, cathepsin D and B in early endosomes in a human B-lymphoblastoid cell line. Kinetic data argue against the possibility that early endosomes are sites of antigen processing and class II association¹⁵. Analysis of other B-lymphoblastoid cell lines using the methods of the present study are required to resolve this discrepancy.

Our steady-state images (see Figs 1*Ab*, 2*f* and *g*, 4*a*, 5*a*, and Table 1) suggest that if recycling of class II molecules occurs in JY cells it is either a minor or very rapid process. MHC molecules are largely excluded from coated pits (Fig. 1*b*). Biochemical analysis of the JY cell line, carried out in the absence of any pharmacological agents that could disturb membrane traffic, failed to show evidence for recycling⁷. The data of Davis

and Cresswell²⁷ showing lack of spontaneous internalization of class II molecules in another human B-lymphoblastoid cell line, agree well with those of our laboratories. Using a different labelling protocol, however, Reid and Watts observed rapid internalization and recycling of MHC molecules, but did so in primaquine exposed EBV-transformed B cells⁴².

Invariant chain, which contains a topogenic signal for delivery to the endosomal system⁴³, was detected in ER, Golgi complex and TGR only. A rapid loss of immunoreactivity, as can be produced by proteolytic cleavage⁴³⁻⁴⁵ of the molecule, may explain our failure to observe invariant chain in the MIIC. An important question is how class II molecules exit from the MIIC identified in this study and reach the cell surface. Is binding of peptide required? What is the role of the invariant chain⁴⁶? An issue that remains to be addressed relates to the traffic of MHC molecules and antigen destined for processing and presentation in other antigen-presenting cells. The steady-state distribution of MHC molecules in different subcellular compartments in monocytes, macrophages, interdigitating dendritic cells, veiled cells and Langerhans cells deserves close examination to uncover a possible relationship with their antigen-presenting capacity. □

Received 28 October; accepted 19 December 1990.

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SUPPLEMENTARY INFORMATION. Requests should be addressed to the London editorial office of *Nature*.

ACKNOWLEDGEMENTS. We thank the photography section of the department of the Laboratory of Cell Biology (University of Utrecht); H. Clevers, J. Peute, J. W. Slot and W. Stoorvogel for critically reading the manuscript; our colleagues, in particular J. W. Slot, H. J. van der Donk and M. Gehrman, for stimulating discussions during this study; and the researchers who supplied us with antibodies. V.O. is supported by the Netherlands Organization for Scientific Research (NWO).

LETTERS TO NATURE

Are BL Lac objects too large to be gravitationally lensed?

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BL LACERTAE objects are extragalactic sources, generally of low redshift, with highly variable, strongly polarized continuum emission ranging from radio wavelengths to X-rays, but with little or no evidence of line emission. It has been suggested¹ that BL Lacs are in fact more distant radio-loud, optically violently variable (OVV) quasars whose continuum is enhanced, relative to the line emission, by gravitational lensing caused by a star in an intervening galaxy. I argue here, however, that the spectral similarity of BL Lacs and OVVs over a wide wavelength range can be explained by gravitational lensing only if the continuum-emitting region is small, a requirement that is contradicted by independent observations.

BL Lac objects are characterized by strong, flat-spectrum radio emission, steep optical/infrared spectra, an absence of (or very weak) emission lines, high polarization and large-amplitude variability on short timescales at all wavelengths from radio through to X-rays. The redshift (z) distribution of BL Lacs is skewed very strongly to smaller z than that of quasars and in fact many BL Lacs are found within elliptical galaxies at $z \leq 0.3$. OVV quasars have very similar continuum properties (in fact they are frequently combined as a single class and called 'blazars'), but they also exhibit the usual QSO (quasi-stellar object) emission lines and the normal QSO redshift distribution, increasing in space density with increasing redshift.

Ostriker and Vietri¹ have suggested that apparently low- z BL Lacs may in fact be more distant OVV quasars undergoing gravitational 'microlensing' by a star in the intervening galaxy in which the BL Lac apparently resides. The two crucial elements in their argument are (1) that, based on variability timescales, the continuum-emitting region of an OVV quasar (and therefore a BL Lac) is small enough (10^{-3} pc) to undergo microlensing by an individual star in a galaxy, whereas the line-emitting regions (of order 1 pc across) are too large; and (2) that, because the probability of lensing increases as the fourth power of the velocity dispersion within the galaxy, microlensing is far more likely to occur within an elliptical than a spiral galaxy.

Recently a small number of BL Lacs have been found to have weak emission lines at much higher redshift than the galaxy around them^{2,3}. Ostriker and Vietri⁴ have argued that this is strong evidence in support of the lensing hypothesis. Narayan and Schneider⁵, however, have pointed out a possible problem in that all the candidate microlensed BL Lacs found so far have only single images, implying that either the core radii of the

lensing galaxies are unusually large (≥ 2 kpc) or that the surface density is unusually low. Peacock (personal communication) has also argued that for there to be a reasonable probability of microlensing, the velocity dispersion and/or the surface density of the galaxy must be large enough that macrolensing should also occur, and we should therefore see several images. I argue here that there is also a problem with Ostriker and Vietri's first assumption concerning the size of the emitting region.

All BL Lacs are radio-loud, with flat radio spectra through to millimetre wavelengths⁶⁻⁹. Furthermore, there is no evidence

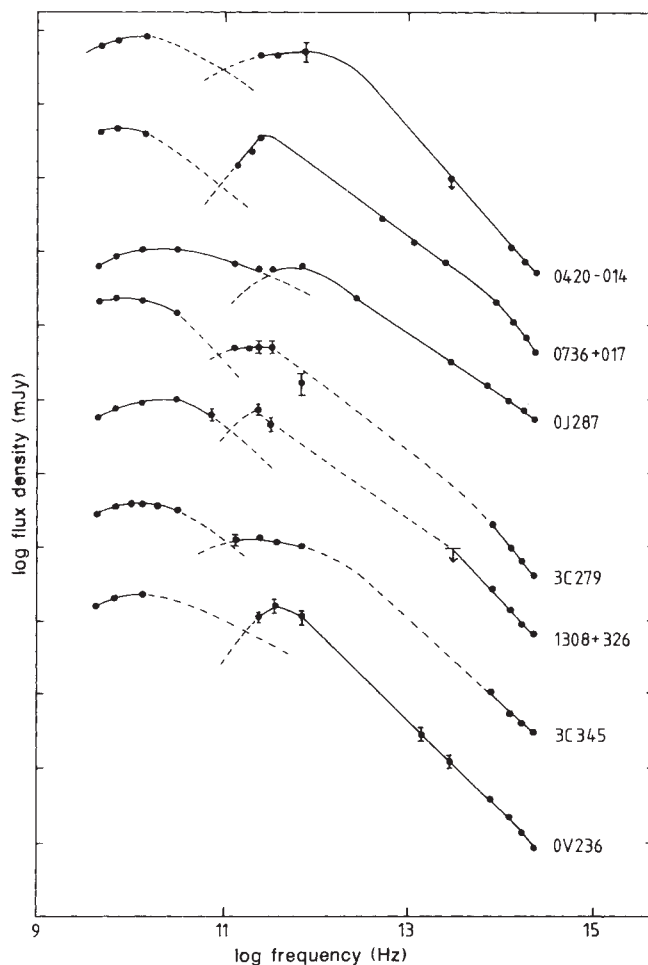


FIG. 1 Quasi-simultaneous radio-infrared spectra of BL Lacs 0420-014; 0J287, 1308+326 and 0V236 and the OVV quasars 0736+017, 3C279 and 3C345, showing the separate millimetre-infrared component, which varies in unison. Figure taken from Brown *et al.*¹⁰.

for differences between the radio-through-optical spectra of BL Lacs and OVV¹⁰⁻¹². (A recent radio-millimetre study of a larger sample of BL Lacs and OVVs specifically to address this point has reached the same conclusion¹³). Over the past few years, very clear evidence has accumulated, based both on spectral shape^{10,12,14} (see also Fig. 1) and coordinated variability^{15,16}, that the millimetre-optical spectra of BL Lacs and OVVs arise in a single region. It has also been shown that radio flares originate in the optical-infrared-millimetre region before evolving to centimetre wavelengths¹⁵⁻¹⁹. It is worth noting that one of the sources on which these conclusions were based was the prime lensing candidate 0235+164.

Any model of the BL Lac phenomenon must therefore provide a unified picture of the origin of at least the optical-radio emission (the X-ray emission may in general arise from a different source but is almost certainly related to the compact millimetre emission during flares^{20,21}). For the lensing model to be valid, therefore, the whole radio-optical spectrum would have to be lensed, which implies a source region smaller than 10^{-3} pc.

There is, however, strong evidence that the emitting region is larger than this. The observed millimetre turnover represents the self-absorption turnover of the most compact region of the source, as the spectra are clearly optically thin above that point. Taking the normal brightness temperature limit, from Compton scattering, of 10^{12} K, simple synchrotron theory (see, for example, ref. 22) yields a typical size for this region of 0.02–0.1 pc, for a typical bulk Lorentz factor of 10 and a peak flux of 10 Jy at around 100 GHz. Although this result should be regarded as approximate, because it assumes both homogeneity and spherical geometry, it is unlikely to be incorrect by orders of magnitude. Very-long-baseline interferometry (VLBI) studies²³ have also revealed structure on parsec scales in several BL Lac objects. It therefore seems very unlikely that the lensing model gives the correct explanation for the excess of BL Lacs at low redshifts, although of course it may apply in a few special cases.

At first sight the assertion that the radio-optical emission arises in a single region may seem inconsistent with the well established observational result that variability timescales are shorter at higher frequencies. But this can be easily understood in terms of the commonly accepted picture of the origin of the continuum emission from compact radio sources: these are generally interpreted as being narrow relativistic jets, aligned at a small angle to our line of sight, with the 'knots' observed by VLBI being shock waves travelling along the jet^{20,24,25}. Because of the extreme variability and frequent occurrence of apparent superluminal motion of the knots away from the core, blazars are usually regarded as being aligned such that the jet is pointed at a small angle to our line of sight. Figure 2 illustrates the basic geometry of all shock-in-jet models. In this picture the effective thickness of the shocked region at any given frequency is determined by the lifetime of the electrons radiating at that frequency; this is limited by their radiation losses, which are greater at higher frequency. Thus variability timescales in the optical can be shorter than in the radio, even though the emission at both wavelengths is coming from the same region²⁰.

It has been pointed out²⁰ that in any shock-in-jet model the effective thickness of the shocked region (which in this case will determine both the minimum variability timescale and the observed self-absorption turnover) could be as little as 10^{-3} to 10^{-4} pc whilst being as much as several parsecs away from the origin of the jet, and that the shocked region could therefore be very much wider than it is thick (Fig. 2). The width will be determined by the opening angle of the jet (for example, for 5° at 1 pc, it will be 0.1 pc). For gravitational microlensing in this case it will be the width and not the thickness that is important, so we predict that the shocked region will not be microlensed by stars in an intervening galaxy. Thus the canonical model of compact radio sources is consistent with the conclusions drawn

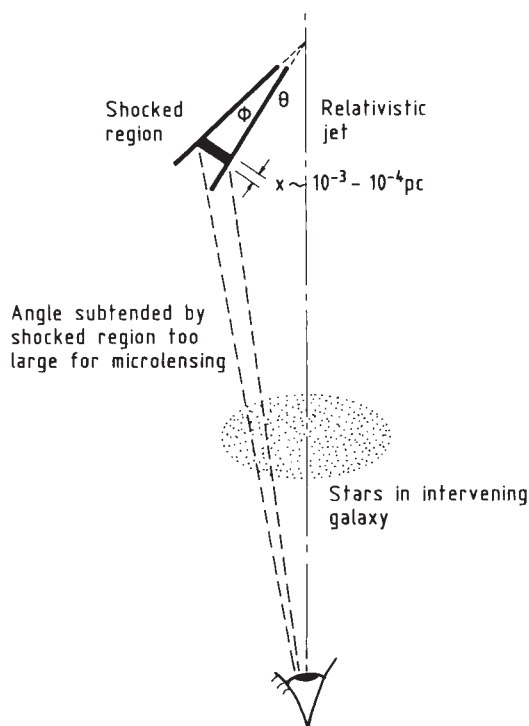


FIG. 2 A schematic of the shock-in-jet model of the continuum emission from BL Lacs. The jet has a small opening angle ϕ and is viewed at a small angle θ to our line of sight. The angle subtended by the shock width is too large for microlensing by an individual star in the intervening galaxy to occur.

above, based on observational results, and the lensing model can be ruled out.

If, as I have argued, microlensing is not significant for the general population of BL Lacs, an alternative solution is still required for the basic problems that prompted Ostriker and Vietri's model, namely the discrepant redshift distribution and the physical interpretation of the lack of emission lines in BL Lacs. Other models may explain these phenomena; for example, Stocke²⁶ has suggested that the evolution of quasars is driven by evolution in the density of the intergalactic medium, and that above a critical value, quasar broad-line gas would be either stripped or evaporated, leaving radio galaxies or BL Lacs, depending on the viewing angle of the radio jet. Another recent model²⁷ associates the low-redshift BL Lacs with the high-redshift OVVs, but suggests that the beamed optical component in these sources evolves cosmologically more slowly than the unbeamed optical emission that dominates in steep-spectrum radio sources. Clearly a great deal more work will be required before the BL Lac phenomenon can be regarded as truly characterized, let alone fully understood. The much larger samples of BL Lacs that are becoming available from X-ray surveys should allow considerable progress in this area. $\square$

Received 30 April; accepted 19 December 1990.

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ACKNOWLEDGEMENTS. I thank John Peacock for many helpful comments and suggestions.

Observation of ^7Be on the surface of LDEF spacecraft

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THE Long Duration Exposure Facility (LDEF), an orbiting unmanned satellite, was recently returned to Earth after almost six years in space. From radioactivity measurements, we have found significant quantities of the isotope ^7Be on the leading edge (but only on the leading edge) of LDEF. Although the absolute atmospheric concentration of ^7Be needed to explain this detection is extremely small (10^{-7} atoms cm^{-3}), its concentration at LDEF's altitude (310 km) must be several orders of magnitude higher than in the stratosphere below, where it is produced by cosmic-ray reactions with atmospheric nitrogen and oxygen nuclei. To explain the presence of ^7Be on the surface of LDEF, it must first be rapidly and efficiently transported to high altitudes, and then adsorbed onto the surface of the spacecraft. Neither process had been expected. Our detection may therefore lead to the use of ^7Be as an exo-atmospheric tracer, as well as to studies of surface interactions in space.

The LDEF spacecraft was launched by the space shuttle Challenger on 7 April 1984 into a nearly circular orbit with an inclination of 28.5° and an altitude of 480 km. It was retrieved by the space shuttle Columbia on 12 January 1990 at an altitude of 310 km. Because of its large mass, long space exposure and the wide variety of materials onboard, the LDEF provided a unique opportunity for induced radioactivity studies. These measurements are still in progress and will be reported elsewhere.

The LDEF spacecraft is a twelve-sided cylindrical aluminium structure, 9.1 m long by 4.3 m in diameter (see Fig. 1). It consists of an open grid to which were attached various experiment trays designed to measure the effects of long space exposure on spacecraft materials and components. Throughout its orbital lifetime, the spacecraft was passively stabilized about all three axes of rotation, allowing one end of the spacecraft to point

always toward the Earth, and fixed leading and trailing sides with respect to the orbital motion.

After its return to the Kennedy Space Center, gamma-ray spectra were obtained along each side of the spacecraft using a germanium detector array provided by the Naval Research Laboratory. Measurements were also made of selected components from the spacecraft. The gamma-ray line at 478 keV from the radioactive decay of ^7Be was unambiguously observed to emanate from the leading side of the spacecraft, as shown in Fig. 1. The weaker signal observed from the trailing side of the spacecraft can be traced to the attenuated gamma-ray flux from the leading surfaces.

Individual components were brought to the Marshall Space Flight Center to quantify the residual radioactivity on the LDEF. A high-purity germanium detector inside a low-level background facility was used to obtain spectra of small aluminium and steel samples taken from the leading and trailing sides. In Figs 2 and 3, gamma-ray spectra of two identical aluminium plates and two steel trunnion end pieces taken from the leading and trailing sides of the spacecraft are shown. A clear ^7Be signal was seen on the leading side, with little or no signal above background on the trailing side.

A polished aluminium plate, used as a thermal control surface in LDEF experiment AO114 (ref. 1), was subjected to several tests to determine the depth of penetration and the form of deposition of the ^7Be . The surface was coated with collodion, stripped to remove all loose particles, then wiped firmly with xylene. Less than 10% of the surface activity was removed by this process, indicating that the ^7Be was neither associated with dust particles nor other soluble surface contaminants. An acid etch, without a stable beryllium carrier, removed several tens of micrometres of the aluminium surface, and most of the ^7Be activity (the remainder was assumed to be re-deposited). This suggests that the ^7Be ions are trapped in the metal oxide surface layer, indicative of a chemical interaction with the surface. Such a process was previously unknown, with the exception of the atomic oxygen effect¹.

In Table 1, the measured number of ^7Be atoms per unit area on various spacecraft surfaces is shown. The results are corrected to the retrieval date of 12 January 1990 and for the offset angle from the leading direction. The areal density for ^7Be on the aluminium and steel is the same within the experimental uncertainty, and is apparently not a strong function of the type or surface condition of the metal. The Teflon thermal coating, however, which was used on many LDEF experiment trays, has a density of ^7Be an order of magnitude lower than that found on the aluminium surface. The reason for this apparent difference in uptake efficiency is unknown, but could be related to the material's covalence-bond structure. The explanation may be complicated, also, by the observed erosion of the Teflon surface by atomic oxygen.

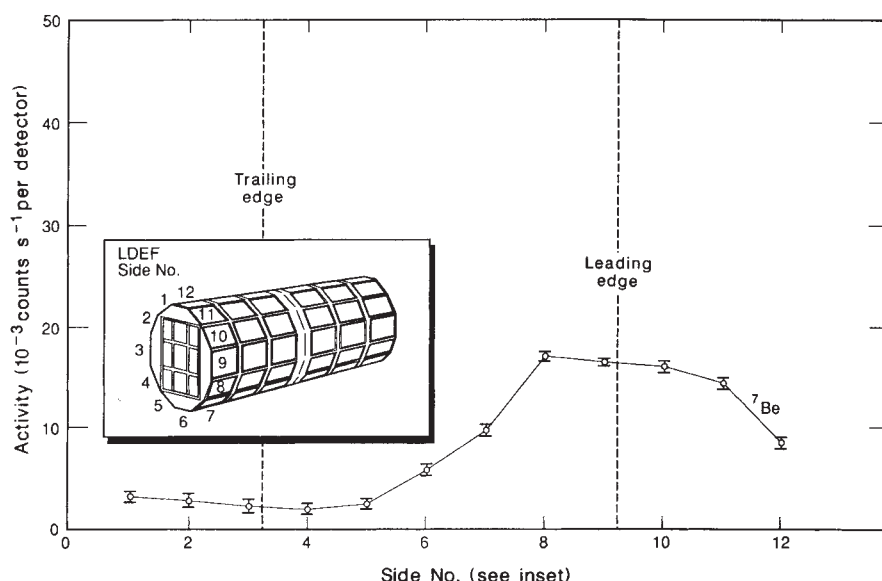
The appearance of ^7Be on the leading surfaces, as shown in Figs 1–3, rules out direct production of the isotope within the spacecraft by the incident radiation flux. In striking contrast to the distribution of ^7Be , the increased flux of geomagnetically trapped protons from the west in this type of orbit² results in higher spallation-induced activities on the trailing side of the spacecraft (see Figs 2 and 3). Such induced activity is also not

TABLE 1 LDEF ^7Be surface concentrations

Material	^7Be areal density ($\times 10^5$ atoms cm^{-2})
Stainless steel trunnion face	5.3 ± 0.7
Polished aluminium plate Exp. AO114	6.7 ± 1.0
Anodized aluminium experiment tray clamp	4.6 ± 0.5
Teflon thermal cover	0.9 ± 0.2

Corrected for decay since recovery and for surface orientation relative to spacecraft ram direction.

FIG. 1 The activity of ^7Be measured from the twelve sides of the LDEF spacecraft at the Kennedy Space Flight Center, corrected to the date of retrieval.



confined to the surface. Although we do observe a small ^7Be signal from aluminium samples taken from the trailing side of the spacecraft, this contribution is at a level we expect from reactions with the incident flux, and is about two orders of magnitude smaller than the surface activity of the leading side. This leads us to conclude that the ^7Be must have accumulated

on the spacecraft surfaces from the ambient atmosphere at orbital altitudes.

The short-lived isotope ^7Be was first detected in the atmosphere by Arnold and Al-Salih in 1955³, and later mapped by others as a function of altitude and latitude⁴⁻⁸. It is produced in the atmosphere by high-energy cosmic-ray interactions with air as are other radioisotopes such as ^{14}C and ^3H . Once formed, ^7Be ions are presumed to oxidize rapidly and attach to small aerosol particles, providing a downward transport mechanism from peak production regions of the atmosphere⁹⁻¹⁶. The primary removal process for ^7Be , which occurs on a timescale comparable to its mean lifetime (~ 77 days), is the washout of the aerosol-attached ^7Be in rain water³⁻⁶.

At a given latitude above ~ 20 km, the production rate of ^7Be varies vertically in proportion to the oxygen-nitrogen gas density. Peak production per unit volume occurs in the lower stratosphere, at ~ 20 km, below which the cosmic-ray flux is substantially attenuated. At higher altitudes, the number of ^7Be atoms produced per unit volume decreases rapidly, but the number of ^7Be atoms per unit mass of air, or concentration, should be essentially constant. Balloon and aircraft measurements^{6,15} are in approximate agreement with this, although few measurements extend much above the peak production altitudes.

We can calculate the concentration of ^7Be at 310 km from the data in Table 1, assuming the trapping efficiency on the metal surfaces is near unity. Using the LDEF orbital velocity of 7.8 km s^{-1} and the 77-day mean lifetime of ^7Be , we find a density of 1.1×10^{-7} atoms cm^{-3} , or a relative concentration of 3.8×10^6 atoms per gram of air. In the peak production region, at altitude 20 km, previous measurements⁴⁻⁸ yield a concentration of 10^3 ^7Be atoms per gram of air, or ~ 0.1 atoms cm^{-3} , in agreement with a simple calculation using the known cosmic-ray flux. Thus, the measured concentration of ^7Be per unit mass of air at 310 km is three to four orders of magnitude in excess of the concentration at 20–50 km.

The concentration of ^7Be in the 300-km range, far in excess of what can be produced in air at that altitude, is evidence of an unknown mechanism transporting ^7Be from the stratosphere. Although the amount being transported (or removed) from the peak production regions is a minute fraction of the total atmospheric burden of ^7Be , the observed density is contrary to assumptions of conventional atmospheric transport and mixing, and in particular, to the assumed attachment to aerosol particles¹⁶. Whatever the mechanism, however, the transport to orbital altitudes must take place on a timescale similar to the mean lifetime of ^7Be .

Systematic low-level induced radioactivity measurements of

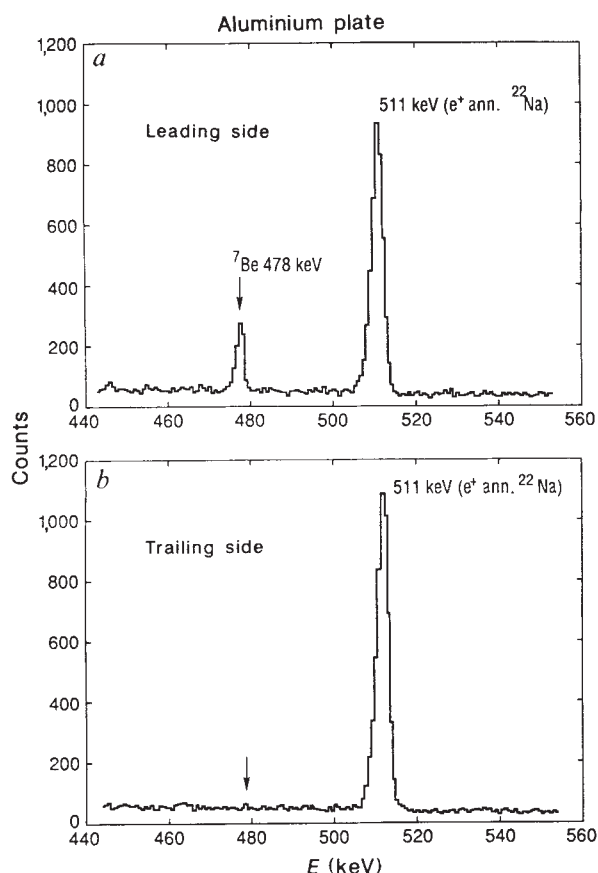


FIG. 2 Gamma-ray spectra of aluminium plates on the leading and trailing sides of LDEF. The isotope ^7Be is identified by the strong line at 478 keV seen on the leading side but not on the trailing side. The strong line at 511 keV is produced by positron-annihilation gamma rays from the spallation product ^{22}Na , as well as laboratory background. Both spectra represent 48-h counts.

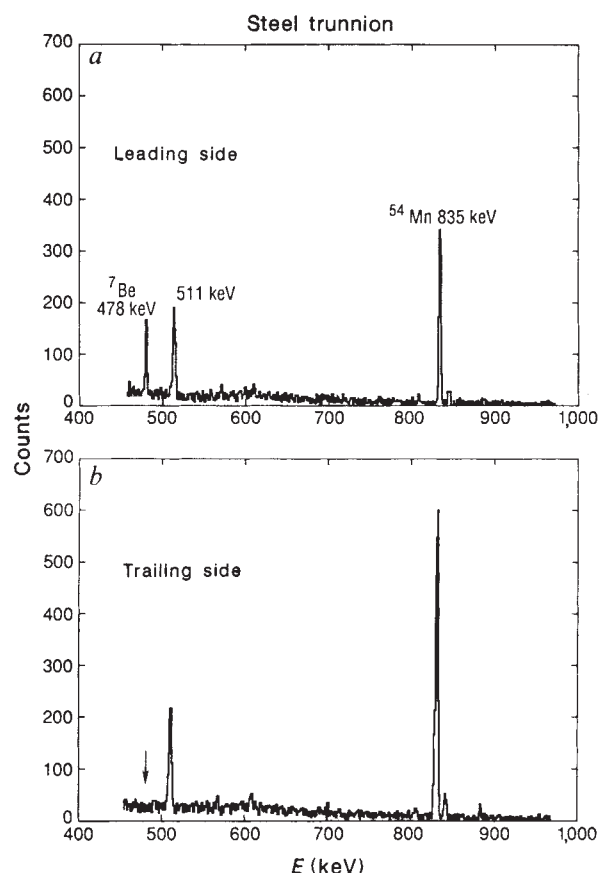


FIG. 3 Gamma-ray spectra of two stainless steel trunnion pin-end pieces from the leading and trailing directions of the spacecraft. As in Fig. 2, the ^7Be isotope is seen only on the leading side of LDEF. The 835-keV line from the spallation product ^{54}Mn is observed on both sides of the spacecraft, but is stronger on the trailing side due to increased proton flux from the west. Both spectra represent 12-h counts.

LDEF materials have not revealed other nuclides with similar surface segregation behaviour; in particular, we have not found the heavier ones that would suggest a meteoritic origin¹⁷. Whereas ^7Be will decay in orbit, there are other non-radioactive light isotopes produced by cosmic rays with similar altitude distributions. Their concentrations should be much higher than that of ^7Be , although more difficult to measure. We are currently attempting to detect other atmospheric cosmic-ray-produced isotopes on LDEF surfaces, such as ^{14}C and ^{10}Be , using accelerator mass spectrometry (A. J. T. Jull, personal communication).

The use of satellite surfaces to sweep up rare atmospheric species may prove to be a new method of investigating atmospheric mixing processes at orbital altitudes. □

Received 16 July 1990; accepted 4 January 1991.

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ACKNOWLEDGEMENTS. We are grateful for the outstanding support of the LDEF Project Office, NASA Langley Research Center and in particular, the LDEF Project Scientist, W. Kinard.

Low surface resistance in $\text{YBa}_2\text{Cu}_3\text{O}_x$ melt-processed thick films

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A LOW surface resistance, R_s , is the key to successful development of radio-frequency and microwave applications of high-temperature superconductors. Here we report the R_s of $\text{YBa}_2\text{Cu}_3\text{O}_x$ thick films on yttria-stabilized zirconia substrates at frequencies up to 50 GHz. Films processed below the peritectic temperature are fine grained, have R_s similar to bulk $\text{YBa}_2\text{Cu}_3\text{O}_x$, generally have low critical current density (J_c) and exhibit little preferred orientation of crystallographic axes. Films processed above the peritectic temperature exhibit preferred orientation in large spherulitic grains, have higher J_c and far lower R_s . For these films the crossover frequency at which R_s equals that of copper is 50 GHz, a factor of two higher than the best bulk material or thick film yet reported and only a factor of ~ 4 lower than high-quality thin films. At the frequencies used for mobile communications (900 MHz and 1.8 GHz), the superconductor losses would be two orders of magnitude lower than those of normal metals. The particular advantages of the thick-film route are the speed and low cost of the process, and the ability to apply the films on curved surfaces and on large areas, the largest so far being $>200\text{ cm}^2$.

Because of the quadratic frequency dependence of R_s in superconductors¹, as opposed to the square-root dependence in normal metals, there is a great incentive to reduce R_s in superconductors so that operation of devices below the cross-over frequency benefit from a lower R_s than that of a normal metal. Measurements of R_s so far can be divided into two broad groups: those made on bulk² or thick-film samples prepared from powder and those on thin films prepared by sputtering, laser ablation or co-evaporation. The thin films, which are usually grown epitaxially with the c axis normal to the plane of the substrate, show losses that are typically lower by two orders of magnitude relative to bulk materials². It is believed that the increased losses in bulk material arise from the weak links between the random array of crystallites which also reduce critical currents³.

Our work was motivated by the need to provide an inexpensive, rapid and effective alternative to thin films for certain applications, and which can be used when thin-film methods are not possible for reasons of size or curvature. The thick-film process also has inherent advantages in that polycrystalline substrates of large size ($\sim 300\text{ mm}$ square) are readily available. The best thick films so far have been formed on silver substrates by electrophoretic deposition^{4–6}, and have an R_s at 21.5 GHz of $18 \pm 3\text{ m}\Omega$ at 77 K. In that work, however, problems arose because of thermal mismatch with the substrate, causing spalling of the films; also, because silver was used as the substrate, the maximum processing temperature could not exceed $\sim 930^\circ\text{C}$. Our technique avoids these problems and gives extremely adherent films which have the lowest R_s for thick films yet reported.

The substrates used were comprised of polycrystalline 3 mol%

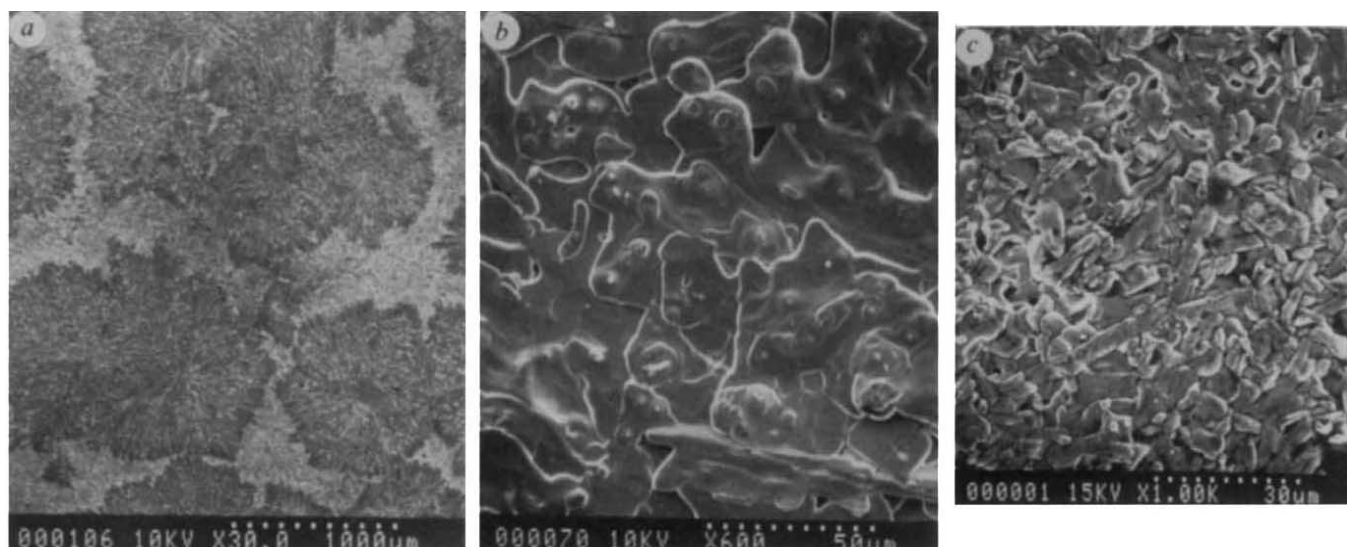


FIG. 1 *a*, Microstructure of thick-film YBCO sintered above the peritectic temperature. Note the large grain size and the spherulitic growth morphology. R_s at 18.5 GHz and 77 K is 6 m Ω . *b*, As *a*, at a magnification that makes it

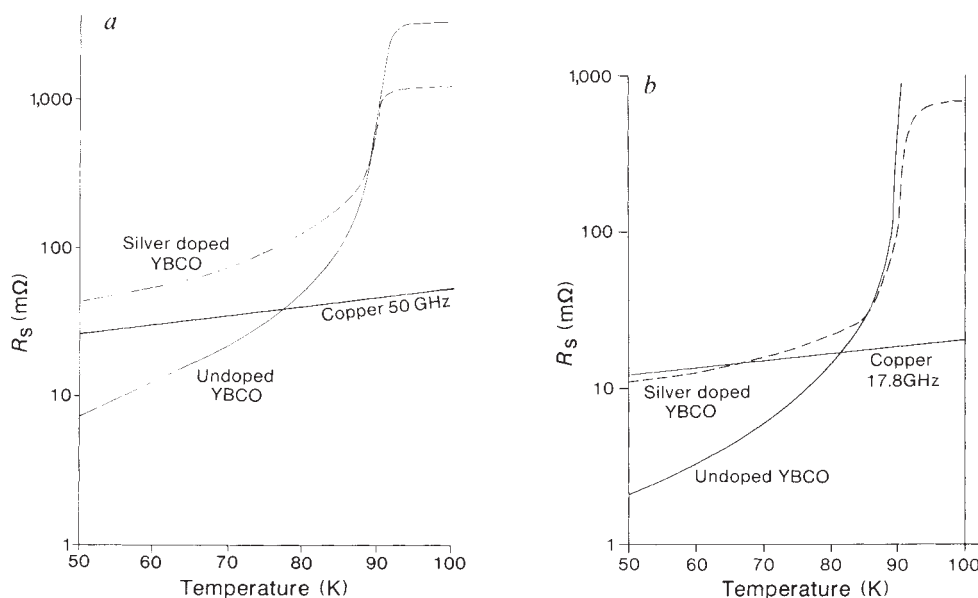
easier to compare with *c*. *c*, Microstructure of thick-film YBCO sintered below the peritectic temperature. R_s at 18.5 GHz is 80–90 m Ω .

yttria-stabilized zirconia, prepared by viscous processing techniques. This involves mixing the ceramic powder with polymers and solvents until a plastic consistency is obtained, and then pressing or rolling flat the material and cutting off substrates of the desired dimensions. The substrates were sintered at 1,450 °C for 2 h, producing a density close to that expected theoretically. The $\text{YBa}_2\text{Cu}_3\text{O}_x$ (YBCO) ink was prepared using standard thick-film ink preparative techniques, involving the use of a three-roll mill to mix the ink thoroughly. Silver metal powder was added to one batch of ink. Previous work⁷ has established the beneficial effects of doping with silver to increase uniformity of oxidation and current density. The ink was deposited onto the substrates by screen printing or by using a 'doctor blade' technique. The films were then sintered for 6 min in flowing oxygen at temperatures between 1,020 °C and 1,050 °C (above the peritectic temperature for $\text{YBa}_2\text{Cu}_3\text{O}_x$), followed by cooling at 1 °C min⁻¹ to room temperature. The thicknesses of the sintered films ranged between 40 and 100 μm . Further details are given in ref. 7.

The structure of the film was investigated using X-ray diffraction and scanning electron microscopy. For $\text{YBa}_2\text{Cu}_3\text{O}_x$, peritectic

decomposition results in the formation of Y_2BaCuO_5 and a Cu-rich liquid. Thus, during sintering, these films were essentially melt-processed. Melt-processing is known to increase the critical current density in bulk $\text{YBa}_2\text{Cu}_3\text{O}_7$ (refs 8–10) and as a consequence of this process, large grains and fewer grain boundaries are produced. Hein *et al.*⁶ have noted a reduction in R_s with increasing grain size. Alford *et al.*¹¹ showed that a complex relationship exists between sintered grain size, initial particle size and porosity; in particular, R_s is highest at the smallest grain sizes. Both of these studies were concerned almost exclusively with sintered materials, and it now appears that the very large grain structure associated with melt-processing gives lower R_s . The large grain sizes in our samples can be seen in Fig. 1*a* and *b*. This type of microstructure is sometimes associated with a high degree of preferred orientation of the film, with the *c* axis perpendicular to the substrate⁷. Although considerable *c*-axis alignment was observed in some samples that exhibited low R_s , other samples with little preferred orientation also had similarly low R_s . In addition, small amounts of secondary phases resulting from incomplete peritectic recombination on cooling

FIG. 2 *a*, R_s as a function of temperature for undoped and silver-doped YBCO thick films measured at 50.2 GHz. *b*, Same as *a*, but for 17.8 GHz.



were observed in some samples. Figure 1c shows a film processed at 960 °C (below the peritectic temperature). This film displays no preferred alignment of the a - b planes, has a small grain size and many more grain boundaries, and at 77 K and 18.5 GHz has far higher losses than melt-processed materials.

The surface resistance R_s was measured using cylindrical TE_{01n} mode cavities operating at 17.8, 18.5 and 50.2 GHz. These modes were chosen because they do not involve current flow from the side wall to the end walls. One end wall could be either a superconducting sample (10×10 mm at 50.2 GHz, 25 mm diameter at 18.45 GHz or 28 mm diameter at 17.8 GHz) or copper. The other held small pins to displace the normally degenerate TM_{11} mode to higher frequencies, where it did not affect the measurement. Using this measurement technique, the dielectric loss of the substrate material is not a problem, particularly because the penetration length is far smaller than the film thickness. In other resonant structures, such as stripline resonators, the high dielectric loss of the partially stabilized zirconia would be deleterious. For this reason we have developed thin barrier layers which lower R_s from >1,000 mΩ at 77 K and 18.5 GHz for films deposited directly on alumina to ~20 mΩ measured under identical conditions when a barrier layer is used. Work is in progress to reduce this value further.

The resonant frequency, insertion loss and bandwidth were measured as a function of temperature using a swept-frequency scalar analyser controlled by a microprocessor. At 17.8 GHz, the excitation signal was generated directly using a sweeper with a low noise specification, and the inclusion of a harmonic multiplier allowed measurements to be made at 50.2 GHz (in the TE_{012} mode). The measurements at 18.5 GHz were performed on a HP8720 vector network analyser to a minimum temperature of 77 K.

Given that the accuracy of the measurements of the normal-state R_s in the superconducting materials above T_c was limited by the poor signal-to-noise ratio, the measured value of 3 Ω is in satisfactory agreement with the value of 2 Ω calculated for a typical resistivity of 20 μm. At low temperatures, resolution was limited by use of the replacement technique to about 2 mΩ. Corrections for cavity loading were made directly using the measured insertion loss, and the incident power was kept approximately constant during the temperature cycle so that the energy in the cavity increased with Q (the ratio of energy stored to the average power loss). Typically, the peak magnetic field

in the 50-GHz cavity increased from 1 to 8 A m⁻¹ as the temperature was reduced from T_c to 70 K. In contrast to measurements on thin films, the skin depth δ (15 μm for $R_s = 3$ Ω at 50 GHz) and the London penetration depth (2–3 μm at low temperatures) are much less than the film thickness, so that the values quoted for R_s do not require correction.

Figure 2a and b shows R_s at 50 GHz and 17.8 GHz respectively, as a function of temperature for two films, one pure YBCO and the other with 10% added silver. The film containing silver has a lower R_s above 90 K, but at lower temperatures the undoped film has a lower loss, with R_s reaching ~40 mΩ at 77 K and 50 GHz and ~10 mΩ at 77 K and 17.8 GHz. This behaviour is not unexpected because above T_c the silver-doped film has a lower resistivity and hence a lower R_s , but at lower temperatures the silver agglomerations will contribute an additional loss. (Ishii *et al.*¹² found that silver-doped films had a lower R_s at all temperatures, but differences in the way in which silver is added make direct comparison difficult.)

Measurements at 18.5 GHz and 77 K on more than 20 melt-processed $YBa_2Cu_3O_x$ samples gave R_s values in the range 5 to 10 mΩ. Verification of the process and results has been established by colleagues at Birmingham University and at Hirst Research Centre, GEC. These values compare very favourably with $YBa_2Cu_3O_x$ that has not been melt-processed, for which $R_s \approx 80$ –90 mΩ at 18.5 GHz and 77 K. For bulk materials sintered below the peritectic, the frequency dependence has been shown not to be quadratic but to vary as $f^{1.2}$ to $f^{1.4}$ in the range 0.3–5 GHz at 77 K (ref. 13). Within the same frequency range and at the same temperature, this frequency dependence also applies to zirconia wires coated with melt-processed $YBa_2Cu_3O_x$, produced in our laboratories. From the data reported here however, the frequency dependence seems to be $f^{1.5}$ to f^2 in the temperature range 50–80 K and at frequencies between 17.8 and 50 GHz. Given sample variability and inhomogeneity and the limited number of frequencies tested, these results are consistent with the local BCS prediction that R_s should increase quadratically with frequency. The normal-state surface resistance in silver-doped samples scales as the square root of frequency but is too large to be measured on pure $YBa_2Cu_3O_x$ in the 17.8-GHz cavity.

If these results are extended to frequencies used for microwave communications (~1 GHz), the melt-processed thick-film superconductor has $R_s \approx 0.015$ –0.03 mΩ, compared with 3–4 mΩ for

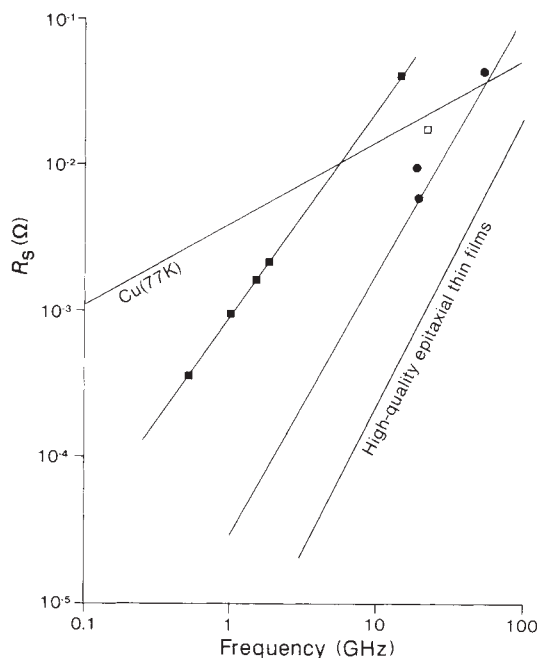


FIG. 3 R_s as a function of frequency for melt-processed thick films (●), electrophoretically deposited thick film⁵ (□) and for YBCO processed below the peritectic temperature (■). Also shown are the values for high-quality thin films.

copper at 77 K. This represents a significant improvement over all other thick-film superconducting materials⁵. This is clear from Fig. 3, which is a plot of R_s as a function of frequency for the present results, material that has not been melt-processed, the best result from electrophoretically deposited thick films^{5,6} and high-quality epitaxial thin films. Thus, melt-textured thick-film $\text{YBa}_2\text{Cu}_3\text{O}_x$ has losses very much lower than those in the bulk material, and at 1 GHz R_s is more than two orders of magnitude lower than copper. These films can be prepared on dimensionally well defined cheap ceramic substrates, avoiding the problems associated with shrinkage that occur with bulk $\text{YBa}_2\text{Cu}_3\text{O}_x$, and also eliminating the need for the expensive, well controlled processes required for the deposition of thin films. □

Received 12 December 1990; accepted 23 January 1991.

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ACKNOWLEDGEMENTS. The work was partly supported by RARDE and RSRE. We gratefully acknowledge the invaluable collaboration with the Superconductivity Research Group at Birmingham University, and the help of Paul Smith at UMIST and Veronique Piro.

X-ray diffraction evidence for aromatic π hydrogen bonding to water

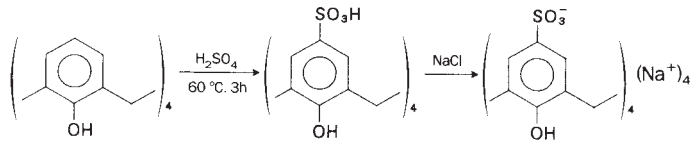
Jerry L. Atwood, Fumio Hamada, Kerry D. Robinson, G. William Orr & Rebecca L. Vincent

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THE interaction of water with aromatic moieties is of importance in biological systems, as most encounter an aqueous environment during their normal functions. For example, common constituents of globular proteins such as phenylalanine, tryptophan and tyrosine possess aromatic side-chains¹ that may encounter water molecules inside the protein structure². As a model for hydrogen-bonding interactions with aromatics, we have performed X-ray diffraction studies on crystalline $\text{Na}_4[\text{calix}[4]\text{arene sulphonate}]\cdot 13.5\text{H}_2\text{O}$. Calixarene molecules^{3,4} contain hydrophobic cavities comprised of aromatic groups, rimmed, in the case of the water-soluble sulphonates ($\text{R} = -\text{SO}_3\text{Na}$), by hydrophilic groups^{5,6}. In the absence of a hydrophobic guest, the cavity invariably contains a water molecule. The low-temperature X-ray crystal structure of this compound (see Table 1 and Fig. 1) shows direct evidence for hydrogen bonding between water and the aromatic π electrons in the solid state.

Complexes of aromatics with small molecules that have hydrogen-bonding capabilities have been the subject of numerous experimental and theoretical studies⁷⁻¹⁰. In particular, weak 1:1 complexes of benzene with HF, HCl or HCN have been studied by pulsed Fourier transform microwave spectroscopy^{11,12}. For $\text{C}_6\text{H}_6\cdots\text{HCl}$, an equilibrium structure was proposed in which all of the π electrons contribute to the bonding interaction. This model provides a $\text{Cl}\cdots\text{centroid}(\text{benzene})$ distance of 3.63 Å, whereas *ab initio* calculations⁷ give a value of 3.81 Å. Related

TABLE 1 Preparation and crystallographic characterization of $\text{Na}_4[\text{calix}[4]\text{arene sulphonate}]\cdot 13.5\text{H}_2\text{O}$

Preparation	
	
X-ray crystallographic data	
Unit cell (−50 °C)	$a = 10.210(2)$ $b = 28.457(4)$ $c = 15.275(2)$ $\alpha = \beta = \gamma = 90$
Space group	$Pnn2$
Z	4
R-factor*	0.063

The structure was solved from single-crystal diffractometer data with the use of direct methods, difference Fourier methods and least-squares refinement of the positional and thermal parameters of all non-hydrogen atoms. Hydrogen atoms on the calixarene were placed in calculated positions, but those on the water molecules were located by difference Fourier techniques and were not refined.

* $R = \sum(|F_o| - |F_c|) / \sum|F_o|$ where F_o and F_c are the observed and calculated structure factors respectively.

calculations⁷ for the π -electron $\cdots\text{H}-\text{O}$ interaction in $\text{C}_6\text{H}_6\cdots\text{H}_2\text{O}$ predict a centroid $\cdots\text{O}$ distance of 3.39 Å with a binding energy of $-3.4 \text{ kcal mol}^{-1}$. As the radius of a chlorine atom is 0.4 Å greater than that of an oxygen atom in a hydrogen-bonded situation¹³, this information leads to an expected experimental $\text{C}_6\text{H}_6\cdots\text{O}$ distance of ~ 3.2 Å.

Matrix-isolation infrared spectroscopic studies¹⁴ have indicated that water forms a weak hydrogen bond with benzene such that effectively the C_6 symmetry of the aromatic remains. The results of two-colour time-of-flight mass-spectroscopic studies¹⁵ have been interpreted as indicating the oxygen atom of water to be centred over the ring at a distance of 3.2 Å, with the hydrogen atoms of the water 3.0 Å above the ring.

Recently, Jorgensen and Severance have used Monte Carlo methods to predict a centroid $\cdots\text{O}$ distance of 3.11 Å and a binding energy of $-3.8 \text{ kcal mol}^{-1}$ in $\text{C}_6\text{H}_6\cdots\text{H}_2\text{O}$, with a geometry in which one hydrogen atom is oriented toward the centre of the ring¹⁶.

Our X-ray diffraction results for the $\text{Na}_4[\text{calix}[4]\text{arene sulphonate}]\cdot 13.5 \text{H}_2\text{O}$ structure (Fig. 1) indicate that the water molecule is imbedded within the cavity of four aromatic groups with centroid $\cdots\text{O}$ distances of 3.16, 4.19, 3.19 and 4.08 Å. The hydrogen atoms are directed toward the nearest centroids, which are opposite one another (see Fig. 1). The centroid $\cdots\text{H}$ separations are 2.38 and 2.50 Å, with centroid $\cdots\text{H}-\text{O}$ angles of 133° and 127°. The $\text{H}-\text{O}-\text{H}$ angle is 125°. The oxygen atom of the imbedded water molecule, W1, completes its approximately tetrahedral coordination with hydrogen bonds to two water molecules, W9 and W10, outside the cavity ($\text{O}\cdots\text{O}$ separations are 2.86 and 2.87 Å). Although the hydrogen atoms of the imbedded water molecule were clearly visible in a final difference electron density map, their positions could not be refined. The evidence for π -electron hydrogen bonding is compelling, however, even if one considers only the locations of W1, W9 and W10: as W1 accepts hydrogen bonds from W9 and W10, the proposed positions of H1a and H1b (that is, in the direction of the π electrons of the nearest two aromatic rings) must be essentially correct.

There are other compounds that exhibit π hydrogen bonding to water. Although the hydrogen atoms have not yet been located, the two $\text{O}\cdots\text{centroid}$ distances are 3.12 and 3.15 Å in $\text{Na}_2\text{K}_2[\text{calix}[4]\text{arene sulphonate}]\cdot 9\text{H}_2\text{O}$, 3.13 and 3.15 Å

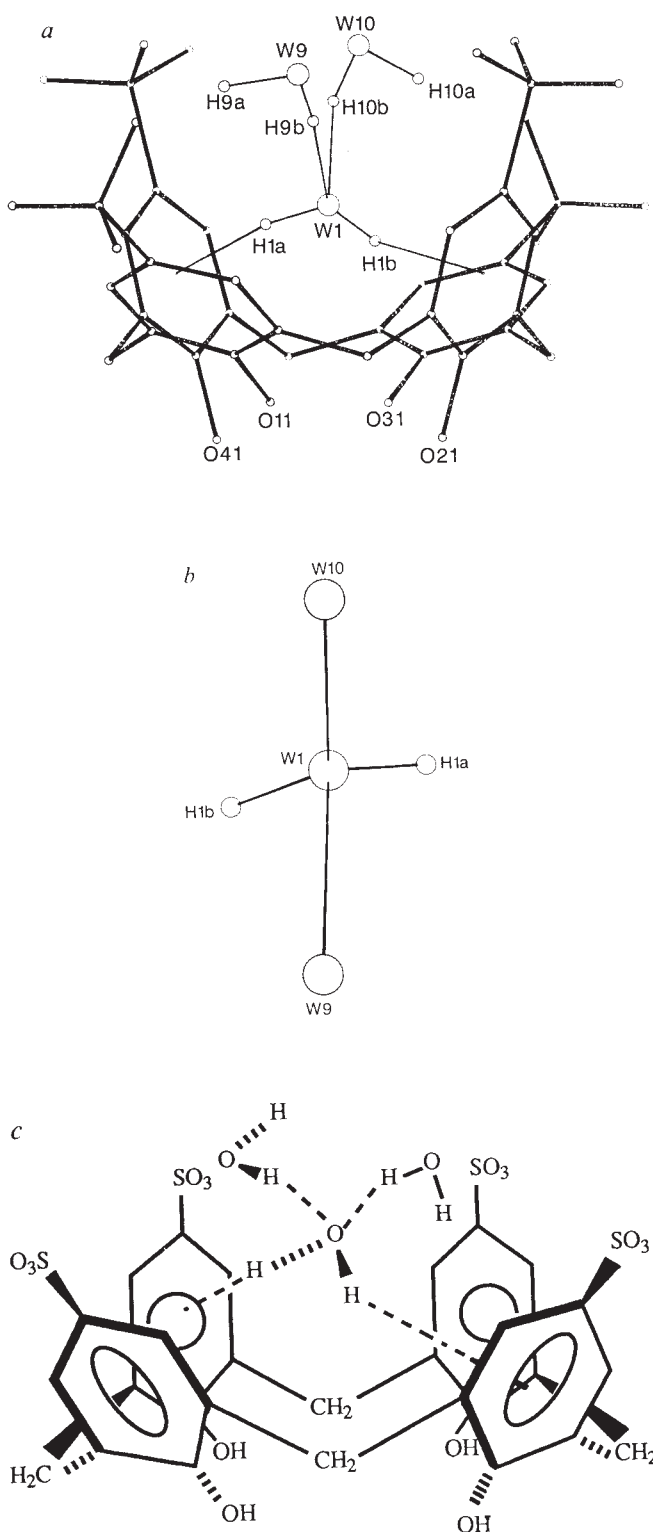


FIG. 1 *a*, The environment of the water molecule, W1, that exhibits π hydrogen bonding to two aromatic rings of the calix[4]arene. The calixarene carries a 4- charge conferred by the four sulphonate groups at the upper rim of the cavity. The phenolic oxygen atoms, O11, O21, O31 and O41, each possess a hydrogen atom. The rings involved in π hydrogen bonding with W1 are associated with O21 and O41. The two water molecules external to the cavity, W9 and W10, are involved in normal hydrogen bonding with W1. *b*, The imbedded water molecule W1 viewed from above W9 and W10 in the direction of the bottom of the calixarene cavity of *a*. The approximate tetrahedral coordination of W1 is emphasized. H1a and H1b are directed toward the π -electron density of the aromatic rings associated with O21 and O41 of *a*. *c*, Schematic illustration of the complete molecular complex.

in $\text{NaK}_3[\text{calix[4]arene sulphonate}] \cdot 9\text{H}_2\text{O}$, and there is one of 3.19 Å in $[\text{methylmorpholinocalix[4]arene}] \cdot 3.5\text{HCl} \cdot 9\text{H}_2\text{O}$. From this series of compounds it is clear that π -electron hydrogen bonding will be found in a wide range of crystal structures. This interaction must also be common in some biological environments. □

Received 30 August 1990; accepted 10 January 1991.

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ACKNOWLEDGEMENTS. We thank the NSF for support.

Synthesis of inorganic nanophase materials in supramolecular protein cages

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THERE is currently great interest in the synthesis of inorganic materials of nanometre dimensions. The small size of these particles endows them with unusual structural and optical properties that may find application in catalysis and electro-optical devices. Such materials may also prove valuable as precursor phases to strong ceramics. Many approaches to the synthesis of these materials have focused on constraining the reaction environment through the use of surface-bound organic groups¹, polymers^{2,3}, porous glasses^{4,5}, zeolites⁶, phospholipid vesicles^{7,8} and reverse micelles⁹. Nanometre-sized particles may also be produced *in vivo* by microorganisms¹⁰. Here we describe a novel synthetic route based on the use of a supramolecular protein structure as a reaction cage in which to form inorganic phases. We show that the iron-storage protein ferritin can be used to generate nanometre-sized iron sulphide particles by *in situ* reaction of the iron oxide core of the native ferritin. Discrete nanoscale particles of manganese and uranium oxo-species can also be formed in the protein cavity. Our results highlight the potential of adapting natural biomineralization processes to problems in materials science, and suggest that the use of biological molecules and their synthetic analogues in mediating solid-state reactions constitutes a promising approach to nanophase engineering.

Ferritin is a robust iron-storage protein that can withstand high temperatures (85 °C) and pH (8.5–9) for limited periods without significant disruption of its quaternary structure of 24 polypeptide subunits. These subunits are assembled into a hollow sphere of 8–9 nm internal diameter¹¹ which contains an iron oxide core of structure similar to that of the mineral ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$). Hydrophilic and hydrophobic channels

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penetrate the protein shell and provide the means by which iron atoms can be accumulated within or removed from the molecules. *In vitro* reconstitution of iron (III) oxide cores can be readily achieved by room-temperature incubation of the intact empty protein (apoferritin) protein with Fe(II) solutions at moderate pH¹².

Figure 1 illustrates the general reaction schemes adopted in our work. Three approaches were undertaken: (a) *in situ* chemical reaction of native iron oxide cores with molecules and ions capable of penetrating into the internal cavity; (b) redox-driven reactions involving metal-ion uptake and deposition in apoferritin molecules; and (c) ion binding and hydrolytic polymerization of metal ions within apoferritin.

Following approach (a), iron sulphide particles were generated within ferritin by reaction of de-aerated solutions of the native protein (Cd-free, Boehringer Horse Spleen Ferritin) in 0.1 M AMPSO buffer (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid), at pH 8.5 with H₂S. A black solution was formed within 20 s. A similar discolouration was observed for the reaction with Na₂S at pH 8.0, except that the formation of iron sulphide was significantly slower. In both cases, no bulk precipitation was observed, indicating that iron sulphide had been produced within the ferritin cavity by reaction of the native iron oxide cores. In comparison, analogous reactions involving synthetically prepared ferrihydrite suspensions resulted in an immediate black discolouration, followed within minutes by a black precipitate. Samples from the ferritin reaction were mounted onto carbon- and formvar-coated-nickel electron

microscope grids and were studied immediately to minimize reoxidation reactions. Analysis was carried out by transmission electron microscopy (TEM), electron diffraction (ED) and energy-dispersive X-ray analysis (EDXA). Whereas the control experiments described above produced gel-like aggregates of amorphous iron sulphide, the protein samples showed discrete electron-dense cores (Fig. 2a) containing iron and sulphur (Fig. 2b). The particles formed in the H₂S preparation were irregular in shape, whereas those formed in the presence of Na₂S were generally spherical. The mean particle size and standard deviation were 6.8 nm and 1.2 nm, and 7.8 nm and 1.1 nm, for the H₂S and Na₂S samples respectively. Before reaction, the ferritin cores were spherical with a mean core diameter of 7.8 nm and standard deviation of 0.6 nm. This suggests that some iron may have been lost from the core on reaction with H₂S. EDXA analysis showed that the Fe/S ratio was close to unity for the H₂S product but was much greater for the Na₂S-derived particles, suggesting only partial reaction in the latter system. This is consistent with the electron diffraction patterns, which showed that some residual well crystallized ferrihydrite (six diffraction lines), characteristic of the original native ferritin core, was present in the iron sulphide cores formed by reaction with Na₂S. In contrast, the electron-dense cores prepared by addition of H₂S gave only three weak, broad ferrihydrite lines, demonstrating considerable loss in crystallinity on reaction. No diffraction lines corresponding to crystalline iron sulphides were observed.

These results indicate that *in situ* transformation of ferritin iron oxide cores to discrete nanometre-sized iron sulphide particles can be readily achieved. Reaction with gaseous H₂S occurs more readily than with Na₂S, the latter giving mixed iron oxide and sulphide cores. This is probably due to more rapid penetration of the protein shell by the neutral H₂S molecules compared with charged HS⁻/S²⁻ species.

Approach (b) was based on the reconstitution of apoferritin molecules with redox-active metal ions such as Mn(II). Apoferritin was prepared by repeated dialysis of ferritin solutions against a 0.5% solution of thioglycolic acid in 0.1 M sodium acetate buffer (pH 4.5), followed by dialysis against 0.15 M NaCl solution. Reconstitution of the protein to a theoretical value of 4,000 Mn ions per molecule was undertaken by combining buffered apoferritin solutions with MnCl₂·4H₂O solutions at pH values of 8.0–9.2. At pH 9, a fine-grained dark brown precipitate was observed in the protein-free control experiment after 4 h whereas the protein solution was a clear light brown colour, indicating specific deposition of manganese oxides within the protein cavity. Experiments at lower pH were left for longer periods (up to two weeks). All reconstitutions showed evidence of some non-specific precipitation outside the protein cage.

Samples for TEM were either centrifuged and dialysed against 0.15 M NaCl or passed down a Sephadex G-25 chromatography column followed by reconcentration using an ultrafiltration cell. Discrete electron-dense particles of regular morphology, with mean size and standard deviation of 7.14 nm and 0.46 nm respectively, were imaged (Fig. 2c). EDXA spectra showed that the cores contained manganese (Fig. 2d). Electron diffraction patterns suggested that the manganese oxide cores were amorphous. By contrast, the non-specific product precipitated outside the protein cavities was identified as a mixture of the crystalline oxides groutite (α -MnOOH) and hausmannite (Mn₃O₄).

Bimetallic cores were prepared by similar experiments involving the successive addition of Fe(II) and Mn(II) solutions to apoferritin at pH 6.5 and pH 8.5 respectively. The resulting cores were discrete nanometre-size particles that contained both iron and manganese (data not shown). Electron diffraction patterns indicated that the Fe and Mn phases of individual cores were present as crystalline ferrihydrite and an amorphous oxide respectively. The influence of manganese on the magnetic properties of the ferrihydrite phase is currently under investigation.

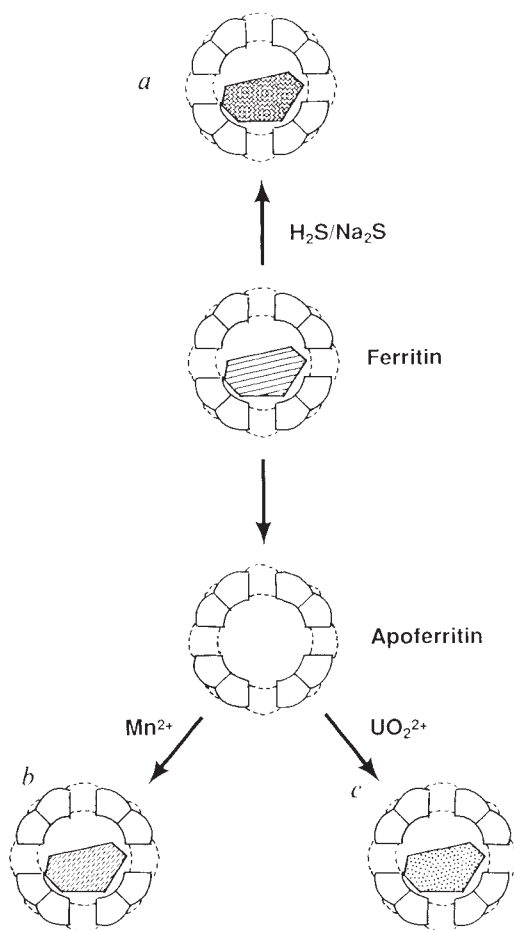
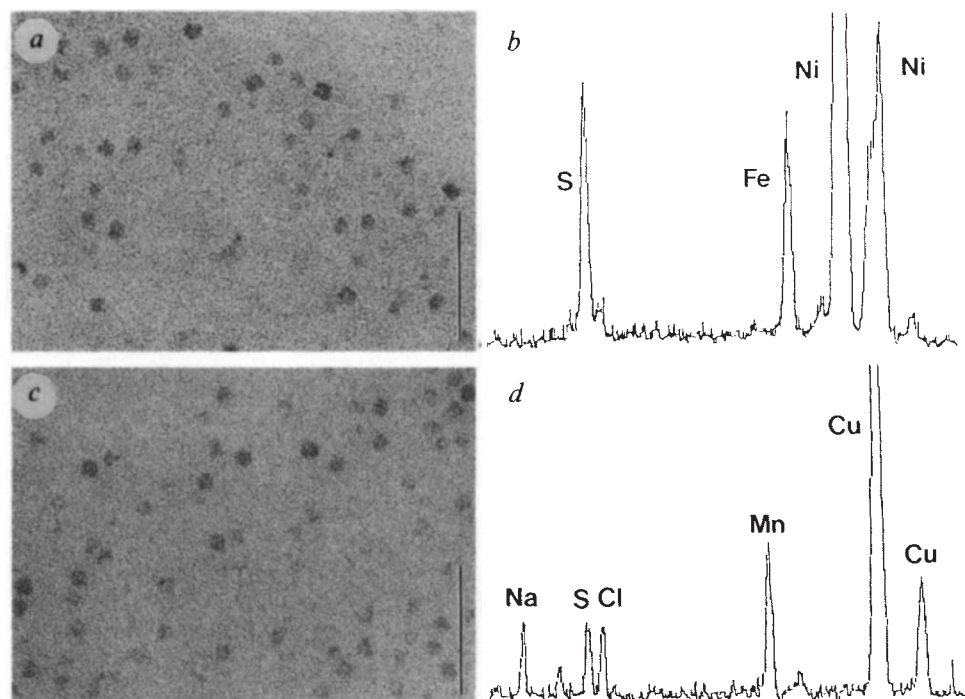


FIG. 1 Schematic representation of the use of ferritin in the synthesis of nanophase materials. a, Iron sulphide formation by *in situ* reaction of native iron oxide cores. b, Manganese oxide reconstitution by redox-driven reactions within apoferritin. c, Uranyl oxyhydroxide deposition by ion-binding and hydrolytic polymerization.

FIG. 2 TEM micrographs and corresponding EDXA spectra of iron sulphide cores (a, b) and manganese oxide cores (c, d) of ferritin. Scale bars in a and c, 50 nm. Ni and Cu peaks arise from the electron microscope grids; Na, Cl and S are background peaks from salt and buffer solutions.



These results indicate that apoferritin molecules can be reconstituted specifically with Mn(II) in a way similar to that commonly used for Fe(II)¹². Mn(II) is known to bind to apoferritin with a stoichiometry of 8 ions per molecule¹³, suggesting an initial binding site in the threefold channel. This site is presumably not involved in oxide deposition, which must involve migration of the manganese cations into the central cavity.

We have conducted similar experiments with other redox-active metal ions such as Ti(III), Co(II) and Cr(II). The results were less conclusive than those for the Mn(II) experiments. TEM analysis showed some evidence of reconstitution with Ti(III) and Cr(II), but the cores were very small and poorly defined. Effective reconstitution of the apoferritin molecules may depend on establishing the appropriate balance between many kinetic processes involving ion uptake, oxidation and polymerization, and factors such as ion concentrations, pH and redox potential may have to be carefully controlled in these reactions.

Finally, we have explored the possibility of using hydrolytic polymerization reactions as a basis for inorganic precipitation within the apoferritin cavity (Fig. 1c). The uranyl cation, UO_2^{2+} , is known to bind to apoferritin with a stoichiometry of 12 ions per molecule¹⁴, possibly suggesting binding at the subunit dimer interface on the internal surface of the protein. A solution of $\text{UO}_2(\text{O}_2\text{CCH}_3)_2$ was added to apoferritin in 0.8 M TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid) at pH 8 in the dark to give a theoretical loading of 4,000 U atoms per apoferritin molecule. A yellow gel was formed after 24 h in both the protein and protein-free experiments. Analysis of the protein sample by TEM showed discrete electron-dense cores of mean diameter 6 nm (Fig. 3a), whereas similar analysis of the control experiment showed no discrete particles. Some cores showed associated staining of their surrounding protein coats by external UO_2^{2+} binding (Fig. 3b). No evidence of crystallinity in the polymerized uranyl oxyhydroxide was obtained.

Thus, supramolecular protein cages have the potential to act as constrained reaction environments in the synthesis of inorganic materials of nanometre dimension. The recent success in expressing human H-chain mutant ferritins¹⁵ provides a possible route to the chemical tailoring of the inner surface of the protein cavity. We plan to investigate the use of such proteins in the oriented nucleation of spatially confined, crystallographically specific nanophase materials. □

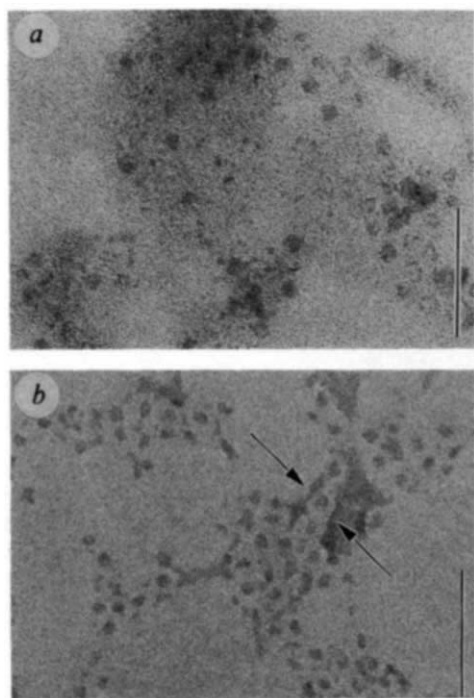


FIG. 3 a, TEM micrograph of apoferritin reconstituted with uranyl acetate, showing discrete cores of uranyl oxyhydroxide. b, As for a, but also showing evidence of negative staining of the external surface of the protein shell (arrows). Scale bars, 50 nm.

Received 13 December 1990; accepted 17 January 1991.

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Static strength and equation of state of rhenium at ultra-high pressures

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YIELDING of materials is not understood well enough for detailed, quantitative predictions of strength to be possible, except by using semi-empirical models^{1,2}. Studies of material strength at high pressures are therefore of fundamental as well as practical interest for determining the relationship between strength and other physical properties^{3–6}. To this end, we have measured the shear stress (τ) supported by rhenium at pressures of up to 120 GPa, far higher than the pressures used in previous studies. Rhenium is of particular interest because it has the highest known bulk and shear moduli among metallic elements^{7–9}. By using two independent methods of determining shear stress at room temperature, we find that rhenium is one of the strongest polycrystalline materials investigated so far, with shear stresses at high pressures reaching $\tau/\mu \approx 0.04(\pm 0.02)$ relative to the shear modulus μ . These values of τ/μ are nevertheless compatible with current theoretical expectations, indicating that the high strength of rhenium is not anomalous^{1,2,6}.

We compressed powdered rhenium (<5–10- μ m grain size, 99.997% purity from Aesar, Johnson Matthey, Inc., Seabrook, New Hampshire) in a modified Mao–Bell type diamond cell¹⁰. The samples were confined by a spring-steel gasket between 1/4 carat diamonds with either 250- μ m or 350- μ m culets. Two runs were carried out with the sample contained in a small amount of pressure medium ($\leq 50\%$ (v/v) 4:1 methanol:ethanol) and a third run was conducted without any pressure medium; as a result, all of our experiments were conducted under conditions of varying, nonhydrostatic stress. Pressure distributions across the sample were determined by the ruby fluorescence method¹¹, and X-ray diffraction patterns were collected with monochromatic Mo K α radiation from a rotating-anode source. The diffraction patterns are collected in an axial geometry, using film that is analysed in a manner described elsewhere¹². The significance of the axial geometry, which is typical for such high-pressure measurements, is that the sample (or unit-cell) volume is determined from diffraction planes that all lie within $<17^\circ$ of the axis of force¹¹.

The pressure (P)–volume (V) measurements obtained at room temperature (Fig. 1a) can be analysed most critically by displaying them as Birch's¹³ normalized pressure F as a function of the Eulerian strain measure f . Here,

$$F = P/[3f(1+2f)^{2/3}] = K_0[1 + a_1 f + a_2 f^2 + \dots] \quad (1)$$

$$f = [(V/V_0)^{-2/3} - 1]/2 \quad (2)$$

with subscript zero indicating zero pressure. The second equality in equation (1) defines the finite-strain equation of state as a series expansion involving the zero-pressure bulk modulus, K_0 , and its pressure derivatives: the first and second derivatives appear in the third- and fourth-order coefficients a_1 and a_2 , respectively^{13,14}.

Figure 1b shows our data compared with a third-order equation of state (300-K isotherm) derived from ultrasonic measurements of K_0 and its first pressure derivative K'_0 for Re (ref. 8). It is evident that all except possibly our highest-pressure data are biased relative to the ultrasonic equation of state, in that we obtain values of F that are too large at each strain f .

To confirm this result, we have reduced shock-wave measurements of the Hugoniot equation of state of rhenium by way of the Mie–Grüneisen formalism^{14,15}. Recent calculations using the first-principles linearized muffin-tin-orbitals (LMTO) method¹⁶ support the use of the Mie–Grüneisen approach for such an analysis (B.K.G. and R.J., unpublished work). In particular, the calculations show that the electronic structure of Re is essentially constant and the electronic contribution to the equation of state is negligible over the range of pressures considered here. In addition, we find that Re is expected to remain crystalline: according to the calculations it melts at much higher shock compressions than those of the data we analyse.

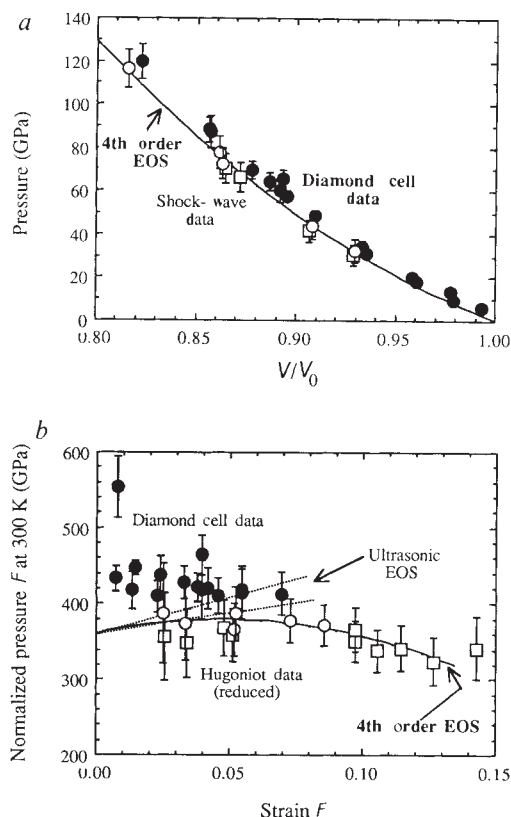


FIG. 1 Equation of state data for Re plotted in terms of volume compression as a function of pressure (a) and Birch's normalized pressure F as a function of the Eulerian strain parameter f (b) (equations (1) and (2)). The solid points are the present diamond-cell data collected at 300 K (one point at $f=0.0023$, $F=880 \pm 44$ GPa lies outside the range of plot b). The open symbols are the shock-wave data of ref. 23 reduced to a 300-K isotherm, with circles and squares corresponding to data collected from samples having initial porosities of 0.2% and 2.3%, respectively^{14,15}. In reducing the Hugoniot data, the Grüneisen parameter was assumed to be given by $\gamma = \gamma_0(V/V_0)^q$, with $\gamma_0 = 2.65 (\pm 0.20)$ and $q = 1 (\pm 1)$ (refs 8, 14, 15, 24). The dashed lines in b indicate the envelope containing the isothermal third-order equation of state derived from the ultrasonic measurements and their uncertainties (ref. 8). The fourth-order fit to the ultrasonic and shock-wave measurements (reduced to the 300-K isotherm) is given by the solid line labelled '4th order EOS'.

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The resulting shock-wave data, reduced to the 300-K isotherm, are included in Fig. 1. In the terminology of refs 14 and 15 these correspond to F_{HT} and f_{3H} , with F_{HT} being approximated by $(K_{0T}/K_{0S})F_{HS}$ (subscript zero, S and T indicate ambient, adiabatic and isothermal conditions, respectively). Also, initial porosity of the samples has been accounted for¹⁵. The data demonstrate that, if anything, a fourth-order equation of state is required, with $a_2 < 0$ (that is, negative curvature in the plot). Note that if any strength effects are present, they should bias the Hugoniot data toward a larger value of a_2 (more-positive curvature, as is found for the static data). Also, the LMTO calculations yield $a_2 < 0$. Our conclusion that a_2 is negative should therefore be reliable.

We combine the ultrasonic and Hugoniot measurements to obtain a fourth-order equation of state defining the 300-K isotherm of Re up to a pressure ≤ 170 GPa ($f \leq 0.1$) (Fig. 1)^{14,15,17}. This is a hydrostatic-compression curve that is given by the isothermal elastic moduli $K_{0T} = 360.3 (\pm 1.5)$ GPa, $K'_{0T} = 5.43 (\pm 0.35)$, $K_{0T}K''_{0T} = -22.2 (\pm 1.5)$, with primes indicating pressure derivatives. It is clear from the figure that all of our nonhydrostatic measurements are biased relative to this equation of state. Specifically, we infer that the volume derived from the X-ray diffraction measurements is systematically too large (f is too small) at a given pressure because of the presence of a large uniaxial component to the stress; that is, significant shear stresses are present in the experiments^{11,18}.

Two observations document the existence of shear stresses in our samples. First, a detailed examination of our diffraction patterns reveals that a preferred orientation, or texturing, is induced in the sample upon compression (Fig. 2). Such textur-

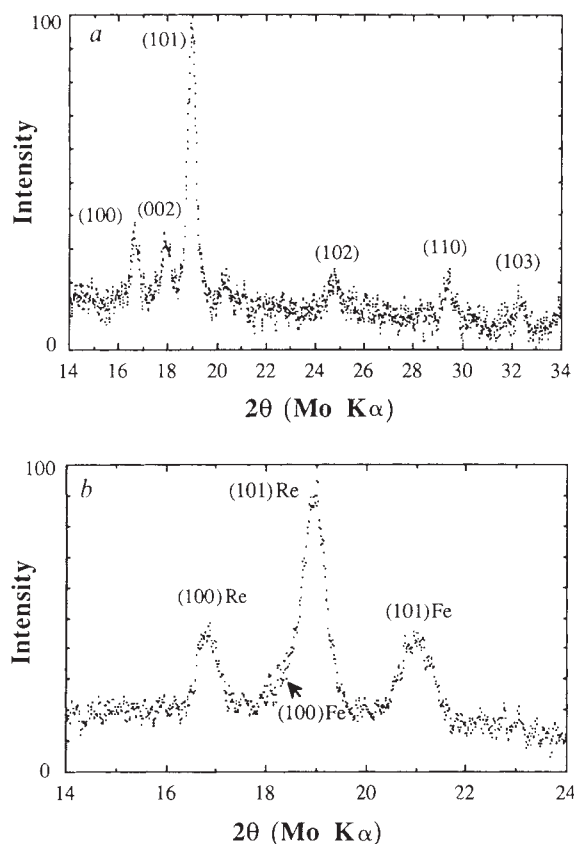


FIG. 2 Powder diffraction patterns of Re in the diamond cell at room temperature. *a*, At 13.2 GPa, we observe all of the diffraction lines expected for rhenium at scattering angles $16^\circ < 2\theta < 34^\circ$. *b*, At higher pressures (60.3 GPa), the (002) line disappears from the rhenium pattern because of preferred orientation in the sample. Diffraction from two of the gasket lines, (100) Fe and (101) Fe, is indicated in the higher-pressure pattern.

ing, which leads to the loss of intensity from the 002 planes, is commonly observed in nonhydrostatically compressed hexagonal-close-packed (h.c.p.) metals (H. K. Mao, personal communication; refs 9, 19 and 20). The shear strain that causes the texturing is a direct consequence of the deviatoric stresses that are present inside the diamond cell^{3,19}.

The second, more quantitative measure of shear stresses is obtained from the maximum pressure gradients observed across our samples, $\partial P/\partial r$, in the radial direction (refs 3 and 21). If σ_1 and σ_3 are the maximum and minimum normal stresses along the axial and radial direction, respectively, the maximum shear stress across the sample is related to the normal stresses and pressure gradient by

$$\tau \equiv (\sigma_1 - \sigma_3)/2 \approx (h/2)(\partial P/\partial r) \quad (3)$$

with h being the sample thickness. Here, stress is taken as positive on compression, and the approximations associated with deriving the second equality in equation (3) have been shown to be valid in practice^{3,4,6}. The resulting estimates of shear stresses in our samples are plotted in Fig. 3 as a function of pressure. The values shown can be considered reliable only to a factor of ~ 2 or better, however, because of the combined uncertainties in our estimates of $\partial P/\partial r$ and h at high pressures (uncertainties owing to the small number of ruby grains in our sample, in the first instance).

From the pressure gradients, we find that shear stresses as large as ~ 10 – 15 GPa can exist in Re at pressures of 100–120 GPa. The actual strength of rhenium, defined as the maximum shear stress that can be sustained by the sample, may be larger yet because we did not attempt to optimize either the geometry of our gasket or the nature of our sample to achieve the highest shear stresses possible³. The presence of the pressure medium in some of the experiments is likely to have reduced the shear stresses, for example. Indeed, the state of stress in our runs is not unlike that found in 'multimegabar' (> 100 – 200 GPa) X-ray diffraction experiments with the diamond cell^{9,22}.

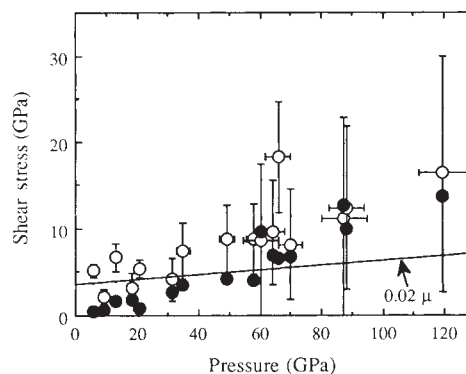


FIG. 3 Shear stress as a function of pressure for static compression at 300 K. Shear stresses (τ) determined from pressure gradients measured across our samples are given as a function of average sample pressure by the solid symbols (for clarity, no uncertainties are shown). Shear stresses inferred from the offset between our diamond-cell measurements and the fourth-order equation of state shown in Fig. 1 are indicated by the open symbols. Note that the pressure gradients must vanish along the central axis of the sample, but this is due to the radial boundary condition: shear stresses are in fact high along this axis and the mean normal stress (average pressure) is not given by the peak pressure³. Thus, even if only the central portion of the sample is probed, for example with a finely collimated X-ray beam from a synchrotron source⁹, the large shear stresses present in nonhydrostatically compressed Re result in a significant offset between the true hydrostat and the compression observed by X-ray diffraction along the central axis of loading^{18,22}. The value of $\mu/50$ is given as a function of pressure for comparison with our data. Here, μ is the shear modulus derived from a third-order Eulerian finite-strain extrapolation of the ultrasonic measurements of ref. 8. Our results are compatible with the strengths measured by Bridgman^{25,26} (shear stresses of 0.8 and 2.0 GPa at pressures of ~ 5 and 10 GPa).

If our analysis of the shear stresses is correct, we can predict the deviation between our X-ray data and the hydrostatic isotherm of Re (Fig. 1). Specifically, the diffraction measures strain in the radial direction (ϵ_3 , from planes that are essentially parallel to the 1 axis), whereas the ruby fluorescence signal yields the pressure $P = (\sigma_1 + 2\sigma_3)/3$ (we average the fluorescence wavelengths observed across the area of our sample)¹¹. If we assume that the sample response is isotropic, such that $\sigma_1(\epsilon_1) = P(f) = \sigma_3(\epsilon_3)$, then the offset between our measurements and the hydrostat can be expressed as

$$\Delta P = (\sigma_1 - \sigma_3)/3 = 2\tau/3 \quad (4)$$

where the second equality is derived from equation (3) (see also ref. 22). Although some texturing is evident in our high-pressure runs, the isotropy of the linear compressibilities of single-crystal Re justifies our assumption of an isotropic sample for the present analysis^{8,9}.

We used (4) to calculate the shear stresses τ required to explain the offset ΔP that we observe relative to the fourth-order finite-strain isotherm derived above. As shown in Fig. 3, these are in good agreement with the values of shear stress obtained from equation (3). Thus, we have an independent confirmation of the remarkably large shear stresses than can be withstood by Re at high pressures and room temperature. For comparison, the highest shear stresses that we have previously observed in similar experiments are 4–6 GPa (refs 3–6). The comparison is all the more striking because the latter values were obtained for oxides (MgO, SiO₂ and Al₂O₃). In contrast, metallic Re would be expected to be more ductile and weaker than these ceramics, yet we find that it is stronger^{1,2}.

Large though these values are for Re, the observed shear stresses are no more than $\sim 2(\pm 1)$ to $4(\pm 2)\%$ of the shear modulus (μ) as a function of increasing pressure. Theoretical expectations are that the maximum value of τ/μ is in the range 0.05–0.10, so the properties of rhenium are compatible with existing models of yielding strengths^{1,2,6}. Nevertheless, because the shear modulus of Re is large ($\mu_0 = 179$ GPa, versus 131 and 160 GPa for MgO and Al₂O₃)⁷ and because $\tau/\mu = 0.04$ is greater than any value that we have previously found for polycrystalline samples at pressure (for example, $\tau/\mu = 0.02$ for alumina to 70 GPa), the absolute values of shear stress sustained across this metal are the highest that we have observed so far^{3–6}. □

New estimates of nitrous oxide emissions from biomass burning

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ATMOSPHERIC nitrous oxide is important because of its role in stratospheric ozone destruction¹ and because it is a greenhouse gas.^{2,3} Measurements indicate that N₂O is increasing in the atmosphere at a rate of $\sim 0.2\%$ yr⁻¹ (ref. 4). Fossil-fuel combustion and biomass burning^{5,6} have been considered to be significant global sources of N₂O, but the recent discovery of an artefact producing increased levels of N₂O in combustion gas samples collected and stored in grab bottles before chemical analysis^{7,8} has resulted in the downgrading of fossil-fuel combustion and the questioning of biomass burning as important sources of N₂O^{9,10}. As almost all reported analyses of N₂O produced from biomass burning have involved essentially the same collection and analysis protocols as used in the fossil-fuel studies, this source of N₂O must also be re-examined. Here we report and compare measurements of N₂O made over a large prescribed fire using a near real-time *in situ* measurement technique with measurements of N₂O from simultaneously collected grab-bottle samples. The results from 27 small laboratory biomass test fires also help clarify the validity of earlier assessments. We conclude that biomass burning contributes $\sim 7\%$ of atmospheric N₂O, as opposed to earlier estimates of several times this value⁶.

The 300-hectare prescribed fire at Morley Lake was conducted in the vicinity of Manitowadge ($\sim 49^\circ 15' \text{ N}$, $85^\circ 52' \text{ W}$) Ontario, Canada, on 24 July 1990. It was a centre-fired burning of partially

TABLE 1 Results from *in situ* and grab-bottle analyses for the Morley Lake prescribed fire in Ontario, Canada

Sample	CO ₂	N ₂ O <i>in situ</i>	Bottle N ₂ O 4–8 h	Bottle N ₂ O 10 days	Bottle N ₂ O 21 days
1B*	354	0.321	0.322	0.322	0.323
2B	358	0.323	0.325	0.324	0.324
13B	355	0.323	0.323	0.324	0.323
14B	362	0.321	0.322	0.322	0.321
15B	363	0.322	0.323	0.323	0.323
21B	361	0.322	0.321	0.323	0.322
3F*	512	0.338	0.340	0.341	0.339
4F	556	0.346	0.347	0.350	0.346
5F	540	0.348	0.344	0.344	0.347
6F	551	0.346	0.349	0.348	0.349
7F	639	0.359	0.355	0.615	0.792
8F	514	0.346	0.341	0.347	0.347
9F	501	0.346	0.349	0.348	0.344
10F	444	0.339	0.335	0.334	0.333
11M*	442	0.338	0.336	0.336	0.337
12M	495	0.341	0.344	0.341	0.346
16S*	417	0.328	0.331	0.332	0.332
17S	429	0.329	0.332	0.358	0.402
18S	434	0.334	0.334	0.332	0.354
19S	406	0.329	0.331	0.333	0.340
20S	514	0.339	0.339	0.348	0.359

N₂O concentration are measured in p.p.m.v. Measurement precision = $\pm 1\%$.

*B, background; F, flaming; M, mixed; S, smouldering.

Received 31 August; accepted 7 November 1990.

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ACKNOWLEDGEMENTS. We thank H. K. Mao, A. L. Ruoff and Y. K. Vohra for helpful comments. This study was supported by the NSF and the Institute of Geophysics and Planetary Physics at Lawrence Livermore National Laboratory.

TABLE 2 *In situ* and grab-bottle emission ratios ($\Delta\text{N}_2\text{O}/\Delta\text{CO}_2$) determined for the Morley Lake fire

Sample	<i>In situ</i> N_2O	Bottle N_2O
3F	0.00010	0.00011
4F	0.00012	0.00012
5F	0.00014	0.00012
6F	0.00012	0.00014
7F	0.00013	0.00011
8F	0.00015	0.00012
9F	0.00017	0.00018
10F	0.00020	0.00014
11M*	0.00019	0.00016
12M	0.00014	0.00015
16S*	0.00009	0.00014
17S	0.00010	0.00013
18S	0.00016	0.00015
19S	0.00015	0.00017
20S	0.00011	0.00010
Mean	0.00014	0.00013
St. dev.	0.00003	0.00002

* F, flaming; M, mixed; S, smouldering.

tramped and harvested boreal forest. A Bell 204 helicopter was used for sampling the air above the fire. The basics of the sampling system and protocol are described elsewhere^{11,12}. A gas chromatograph with ^{63}Ni detection was used for *in situ* N_2O analysis during the fire. Simultaneously collected stainless steel grab-bottle samples were analysed from 4–8 h after collection, then re-analysed 10 and 21 days after collection. Additionally, small laboratory fires (~1–2 kg) consisting of loblolly pine branches, twigs and several marsh grasses were burned, sampled and analysed with virtually the same protocols as the Morley Lake fire to allow further comparisons to be made.

The results from the *in situ* analyses and subsequent analyses

of the steel grab-bottle collections from the Morley Lake fire (see Table 1) indicate that no statistically significant differences in the N_2O mixing ratios were found between the *in situ* determinations of N_2O and those for the first analysis of the grab bottles made 4–8 h after collections. But significant growth in N_2O concentrations was observed in several samples after 10 and 21 days of storage.

CO_2 -normalized N_2O emission ratios ($\text{ERs} = \Delta\text{N}_2\text{O}/\Delta\text{CO}_2$ (v/v); where Δ = above background) determined for these samples are shown in Table 2. Again, no statistical differences were found between the emission ratios determined for the near-real-time *in situ* N_2O results and the corresponding grab-bottle samples analysed 4–8 h after collection.

Results from the laboratory fires are presented in Table 3. Fourteen of the 27 samples were found to increase significantly after 10 days of storage. Growth rates and the amounts of artefact N_2O produced correlated well with the initial concentrations. Higher initial N_2O and CO_2 concentrations (presumably other combustion products as well) typically produced more artefact N_2O faster. It is clear from the results given in Tables 1 and 3 that the use of stainless steel grab bottles for the collection and storage of N_2O emissions from biomass fires before chemical analysis presents a significant risk.

Table 4 summarizes the mean CO_2 -normalized emission ratios determined from grab-bottle collections for two fires in chaparral¹³, two fires in Florida graminoid wetlands¹², three fires in boreal forest⁹, 27 small laboratory test fires and the Morley Lake fire. Mean emission ratios determined using the first N_2O analysis for the laboratory fires are about an order of magnitude lower than those determined for the larger fires. That the CO_2 -normalized ERs obtained from the laboratory fires are substantially lower than those determined for the other large-scale fires studied is puzzling. Other researchers have obtained similar results from laboratory fires.¹⁴

We feel obliged to speculate about the significantly smaller ERs determined from the laboratory fires as this result, along with the discovery of the bottle storage artefact, has prompted

TABLE 3 Summary of results from small laboratory fires of loblolly pine (P), marsh grass (G) and equal mixes of both (P/G)

Sample	CO_2 (p.p.m.v.)	t_0	N_2O (p.p.m.v.)							
			$\frac{1}{2}$ h	1 h	6 h	12 h	1 day	3 days	5 days	10 days
1P	3,200	0.402	0.400	0.403	0.403	0.402	0.401	0.403	0.403	0.402
2P	17,900	0.749	0.754	0.789	0.848	0.891	0.937	1.233	1.371	1.811
3P	2,700	0.381	0.379	0.380	0.381	0.382	0.383	0.382	0.383	0.383
4P	6,700	0.533	0.533	0.534	0.536	0.535	0.533	0.561	0.593	0.629
5P	5,200	0.441	0.441	0.443	0.442	0.442	0.443	0.443	0.445	0.445
6P	4,000	0.382	0.384	0.382	0.384	0.381	0.381	0.382	0.381	0.379
7P	10,800	0.677	0.677	0.679	0.684	0.688	0.711	1.011	1.103	1.251
8P	17,800	0.788	0.799	0.887	0.933	0.941	0.973	1.102	1.173	1.213
9P	3,040	0.396	0.396	0.395	0.397	0.397	0.396	0.395	0.395	0.395
10G	9,300	0.601	0.599	0.603	0.602	0.687	0.785	1.122	1.619	2.336
11G	4,700	0.461	0.461	0.463	0.462	0.463	0.463	0.493	0.508	0.549
12G	7,500	0.527	0.526	0.527	0.526	0.527	0.597	0.697	0.728	0.751
13G	1,900	0.348	0.347	0.347	0.347	0.347	0.346	0.351	0.351	0.351
14G	1,870	0.423	0.423	0.421	0.421	0.421	0.420	0.422	0.421	0.421
15G	8,100	0.556	0.554	0.555	0.556	0.557	0.575	0.661	0.725	0.833
16G	4,450	0.449	0.451	0.451	0.451	0.451	0.453	0.474	0.491	0.533
17G	3,700	0.381	0.381	0.379	0.381	0.380	0.379	0.378	0.377	0.381
18G	2,100	0.354	0.353	0.353	0.353	0.351	0.353	0.354	0.355	0.354
19P/G	22,800	0.834	0.856	0.911	1.012	1.044	1.092	1.199	1.213	1.358
20P/G	4,170	0.415	0.415	0.414	0.416	0.414	0.416	0.561	0.593	0.672
21P/G	2,100	0.348	0.348	0.349	0.351	0.349	0.348	0.349	0.350	0.348
22P/G	10,990	0.661	0.661	0.669	0.677	0.759	0.796	0.833	0.877	0.997
23P/G	2,400	0.389	0.388	0.388	0.388	0.385	0.386	0.389	0.395	0.456
24P/G	1,960	0.385	0.385	0.386	0.386	0.387	0.385	0.385	0.384	0.385
25P/G	1,600	0.340	0.340	0.341	0.339	0.341	0.339	0.339	0.339	0.339
26P/G	1,100	0.336	0.336	0.335	0.337	0.335	0.335	0.334	0.336	0.336
27P/G	1,850	0.422	0.423	0.423	0.422	0.423	0.427	0.498	0.537	0.577

All samples collected during flaming combustion; measurement precision = $\pm 1\%$.

TABLE 4 Summary of N₂O emission ratios

System	Flaming (%)	Mixed (%)	Smouldering (%)
Chaparral	0.014 ± 0.003 (9)*	0.021 ± 0.011 (9)	0.039 ± 0.008 (5)
Wetlands	0.019 ± 0.004 (13)	0.024 ± 0.011 (9)	0.025 ± 0.007 (10)
Boreal	0.019 ± 0.004 (15)	0.016 ± 0.002 (11)	0.017 ± 0.002 (12)
Laboratory	NA	0.0024 ± 0.0011 (27)	NA
Morley Lake	0.013 ± 0.003 (8)	0.015 ± 0.000 (2)	0.014 ± 0.003 (5)

* (), number of samples analysed.

questioning of the importance of biomass burning as a primary global source of N₂O. One speculation offered involves potential chemical reactions, particularly heterogeneous condensed-phase reactions. Field collections typically involve sampling aged (older than laboratory) smoke plumes, thereby allowing combustion products more opportunity to undergo heterogeneous N₂O-producing reactions on aerosols. This, in turn, might have led to the higher emission ratios determined for N₂O over the large fires. Furthermore, combustion itself may not be the only source of the N₂O in atmospheric smoke plumes. Significant releases of biogenically produced N₂O could result from the rapid heating of soils during a large fire. Recent measurements indicate significant increases in biogenic emissions of N₂O following burning¹⁵. Thus, the N₂O measured over a fire may consist of the sum of combustion and other N₂O production/release processes.

The bulk of the evidence suggests that the concentrations of N₂O in grab bottles over the concentration ranges that we have normally encountered over large fires will be stable for the first few hours after collection. This is supported by both the small-scale laboratory fires and the large prescribed fire results.

Comparison of the *in situ* and grab-sample results from the Morley Lake fire suggests that the emission ratios we have previously reported in which stainless steel grab bottles were used exclusively for collection may be reasonably free of the N₂O-producing artefact. This is because most of the samples were analysed within a day, and good agreement was generally observed between the mean emission ratios obtained for all the fires sampled, including Morley Lake (see Table 4).

Finally, CO₂-normalized ERs for N₂O in the range of 0.01–0.02% seem to be representative of biomass fire emissions of N₂O from the ecosystems we have studied. Thus, some of the earlier assessments^{9,16} for N₂O emissions based largely on grab-bottle sampling seem not to have been seriously affected by the N₂O-producing artefact.

If we assume a mean value of 2,850 Tg of carbon in the form of CO₂ is generated annually by global biomass burning¹⁷, and an ER for N₂O of 0.015% (as reported here), then ~1 Tg N as N₂O is generated annually by biomass burning. Thus, no more than 7% (and probably less) of the global source of N₂O (estimated to be 13–15 Tg N yr⁻¹)⁶ can be attributed directly to biomass burning. The downgrading of N₂O production from fossil-fuel combustion coupled with the current estimates of N₂O production from biomass burning lead to a significant imbalance in the global budget of N₂O. It is possible that a significant global source of N₂O has yet to be identified, or that known source(s) may have been underestimated. □

Received 23 October 1990; accepted 4 January 1991.

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Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin

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A CENTRAL feature of host defence is the ability of leukocytes to enter tissues in response to immune or inflammatory stimuli. The leukocyte adhesion molecule-1 (LAM-1) regulates the migration of human leukocytes by mediating the binding both of lymphocytes to high endothelial venules of peripheral lymph nodes and of neutrophils to endothelium at inflammatory sites^{1–10}. As lymphocytes and neutrophils express the same LAM-1 protein^{11,12}, it is not clear how lineage-specific differences in leukocyte migration are controlled. We now report that the affinity of LAM-1 for a carbohydrate-based ligand, PPME^{13–16}, is dramatically increased following lymphocyte and neutrophil activation by lineage-specific stimuli. In addition, activation of lymphocytes by physiological stimuli enhanced LAM-1-dependent binding to high endothelial venules. Thus, transient changes in LAM-1 affinity after leukocyte stimulation probably directly influence leukocyte migration.

The LAM-1 molecule (TQ1, Leu-8) contains a lectin-like domain which binds a specific glycoconjugate on target cells^{2–7,13–16}. The interaction of LAM-1 with its natural ligand can be inhibited by specific polysaccharides rich in mannose 6-phosphate, such as polyphosphomonoester core polysaccharide derived from the yeast *Hansenula* (PPME)^{13–16}. PPME can also be labelled and used to assess LAM-1 binding activity without the influence of other adhesion receptors¹⁷. Both lymphocytes and neutrophils bind fluoresceinated PPME; monoclonal antibodies reactive with LAM-1, which inhibit lymphocyte binding to HEV¹⁸, also inhibited PPME binding (Fig. 1). EDTA also inhibited PPME uptake, consistent with involvement of the calcium-dependent lectin-like domain of LAM-1. Therefore, PPME provides a specific soluble ligand to investigate LAM-1 expression and function.

We have examined whether LAM-1 function is regulated by lineage-specific stimuli that would direct the homing of different cells to different tissues. The interaction of LAM-1 with PPME was investigated using T cells before and after activation by crosslinking the T-cell receptor complex or through the CD2 pathway¹⁹, and using neutrophils before and after exposure to granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (G/M-CSF) or tumour necrosis factor α (TNF α)^{10,12,20}. Stimulation of T cells and neutrophils resulted in marked enhancement of PPME binding (Fig. 1). Expression of LAM-1 on T cells was similar before and after stimulation, whereas expression on neutrophils decreased after stimulation (Fig. 1c). Activation of lymphocytes consistently increased PPME binding by three- to fourfold as measured using radiolabelled PPME, and also increased lymphocyte binding to high endothelial venules (HEV) (Table 1). Binding to PPME and HEV were blocked (80–95%) by anti-

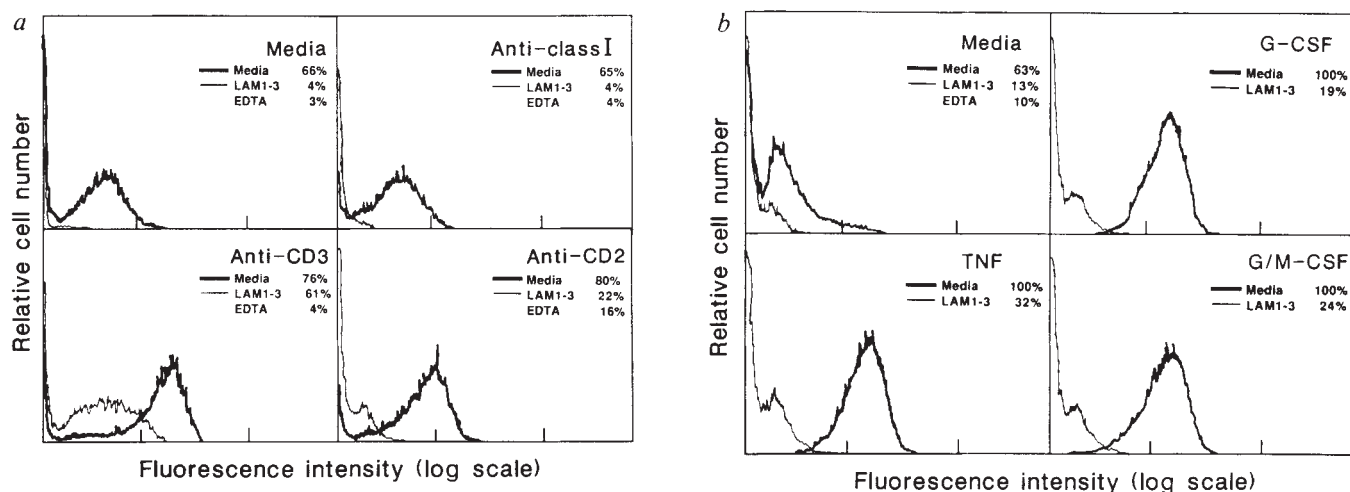
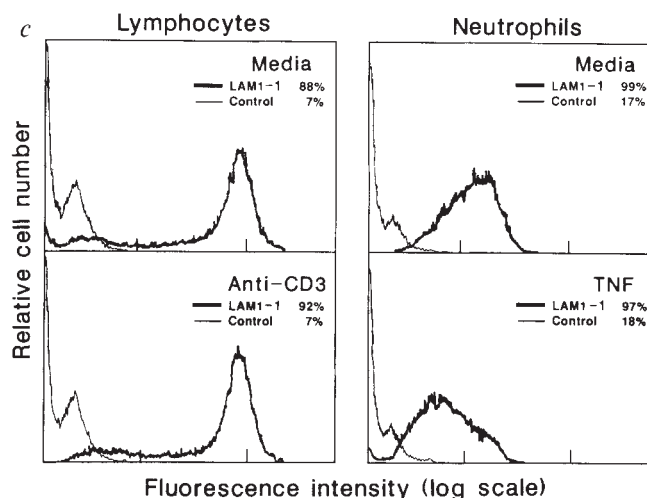


FIG. 1 Leukocyte binding of PPME increases rapidly following activation. *a*, Lymphocytes were stimulated through the CD3 or CD2 pathways for 20 min at 4 °C and their binding of fluorescein-labelled PPME assessed by flow cytometry. Identical results were obtained after activation for 5 min at 37 °C (not shown). PPME binding was inhibited by EDTA or the anti-LAM-1-3 antibody¹⁸ that binds to the lectin domain of LAM-1 (G.S.K., O.S. and T.F.T., manuscript submitted) which is similar to the MEL-14 antibody in mice³⁰. *b*, Neutrophils were stimulated with G-CSF, TNF or G/M-CSF for 5 min at 37 °C and PPME binding assessed as above. Identical results were obtained after activation for 20 min at 4 °C (not shown). *c*, Cell surface expression of LAM-1 did not increase after stimulation as determined by indirect immunofluorescence analysis of cell-surface LAM-1. These data are representative of those obtained in three experiments.

METHODS. *a*, *b*, Blood mononuclear cells were isolated as described³ and incubated for 30–60 min at 37 °C in culture medium before incubation (10^6 cells per $100 \mu\text{l}$) for 20 min at 4 °C with medium or with control anti-HLA class I antibody (W6/32), anti-CD3 (RW2-8C8) or anti-CD2 (anti-T11₂ plus anti-T11₃)¹⁹ antibodies used as diluted ascites fluid (1:250). Anti-CD3 antibody-stimulated cells were washed once and incubated with $100 \mu\text{l}$ goat anti-mouse immunoglobulin diluted in PBS (0.5 mg ml^{-1} ; the crosslinking antibody interfered with anti-LAM-1-3 blocking in some experiments). Neutrophils were isolated using mono-poly resolving medium (Flow Laboratories) and cultured with medium, recombinant human (rh) G-CSF ($1:100$ dilution of conditioned media), 100 U ml^{-1} rhTNF α or 10 ng ml^{-1} rh G/M-CSF as described¹². After washing with ice-cold PBS-BSA, cells were incubated for 30 min at 4 °C with $100 \mu\text{l}$ of fluorescein-labelled PPME ($3 \mu\text{g ml}^{-1}$) as described^{14,18}, washed twice, and the fluorescence intensity of treated cells assessed by flow cytometry. Replicate tubes of cells in buffer containing EDTA (5 mM) or anti-LAM-1-3 were also reacted with PPME. Values represent the percentage of specifically stained cells and



fluorescence is shown on a 3-decade log scale. *c*, Expression of LAM-1 was assessed using phycoerythrin-labelled anti-TQ1 antibody (Coulter Immunology) with labelled mouse IgG used to assess nonspecific staining as described³. Binding of labelled anti-TQ1 by the antibody used to crosslink CD3 antibodies was blocked by adding an excess of mouse ascitic fluid.

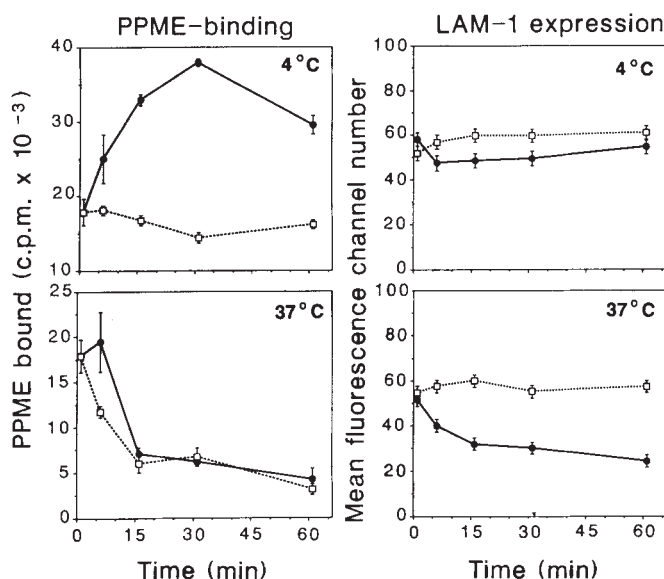


FIG. 2 Enhanced binding of PPME by LAM-1 after lymphocyte stimulation is transient and does not result from an increase in LAM-1 receptor number. Lymphocytes (10^6 per tube) were incubated in medium (□) or were stimulated by T-cell receptor crosslinking (●) as in Fig. 1 at 4 °C or 37 °C. Cells were washed and incubated for 60 min with [^{125}I]PPME ($0.36 \mu\text{g ml}^{-1}$, 2.7×10^5 c.p.m. per tube) in PBS or PBS plus 5 mM EDTA and processed as in Table 1. The values represent the mean c.p.m. ($\pm$ s.d.) of the Ca^{2+} -dependent binding. PPME binding in the presence of EDTA was less than 5% of the values shown and 80–95% of PPME binding was blocked by anti-LAM-1-3. At 37 °C, after an initial increase at 5 min, PPME binding decreased to less than its starting value because LAM-1 is shed during culture^{10,18}, as shown. Flow cytometry, a more rapid assay of PPME binding, demonstrated a significant increase in PPME binding within 5 min of activation at 37 °C (Fig. 1). LAM-1 expression was assessed as in Fig. 1. Values represent the mean fluorescence channel number for staining intensity $\pm$ s.d. (1–256 channels on a 3-decade scale). Background staining was equivalent in all samples and did not change during the experiments. Data shown are representative of those obtained in two experiments.

TABLE 1 PPME and HEV binding are enhanced by lymphocyte activation

Stimulus	Binding of [125 I]PPME (c.p.m. $\pm$ s.d.)			Cells bound/HEV ($\pm$ s.d.)	
	Medium	Anti-LAM-1-3	EDTA	Medium	Anti-LAM-1-3
Medium	4,857 $\pm$ 372	1,160 $\pm$ 104	578	1.4 $\pm$ 0.2	0.2 $\pm$ 0.2
Anti-LAM-1-10	6,868 $\pm$ 571	2,103 $\pm$ 950	2,030	1.5 $\pm$ 0.8	0.2 $\pm$ 0.2
Anti-CD2	17,886 $\pm$ 419	2,863 $\pm$ 689	965	2.4 $\pm$ 0.9**	0.2 $\pm$ 0.2
Anti-CD3	19,718 $\pm$ 1,294	1,211 $\pm$ 618	303	2.7 $\pm$ 1.0*	0.2 $\pm$ 0.2

Blood lymphocytes were isolated, incubated for 20 min at 4 °C with the indicated antibodies, and anti-CD3 was crosslinked as in Fig. 1. After one wash, the cells were incubated with [125 I]-labelled PPME (0.36 μ g ml $^{-1}$, 2.2×10^5 c.p.m. per sample) at 4 °C for 30 min. Anti-LAM-1-3 was added 1 min before the addition of the test antibody and during all incubations. The calcium-independent binding of [125 I]PPME was assessed in the presence of 5 mM EDTA. Cells were washed, resuspended in PBS-BSA and layered on a 750- μ l cushion of 75% (v/v) calf serum. The cell pellet was isolated and bound [125 I]PPME assessed by γ counting. These data are representative of those obtained in three experiments. Fluorescein-labelled PPME was iodinated as described²⁸, the specific activity (2×10^4 c.p.m. ng $^{-1}$) determined by self-displacement curve analysis²⁹, and the maximum binding capacity was 20%. HEV binding was assessed as described³ using 12- μ m freshly cut frozen rat lymph node sections. The number of lymphocytes bound to all HEV was counted on coded slides. Values are means $\pm$ s.d. of four experiments and the differences between control antibody-treated cells and anti-CD2 and anti-CD3 treated cells were significant (* $P < 0.05$, ** $P < 0.01$ using the paired Student's *t*-test).

LAM-1-3 antibody¹⁸ or by EDTA. Binding of other anti-LAM-1 antibodies, or antibodies reactive with HLA class I antigens or CD18, had no effect. Thus, cellular activation led to enhanced binding of PPME by both lymphocytes and neutrophils, which was associated with an increase in lymphocyte adhesion to HEV.

Binding of fluoresceinated PPME by lymphocytes and neutrophils was increased within minutes of addition of the

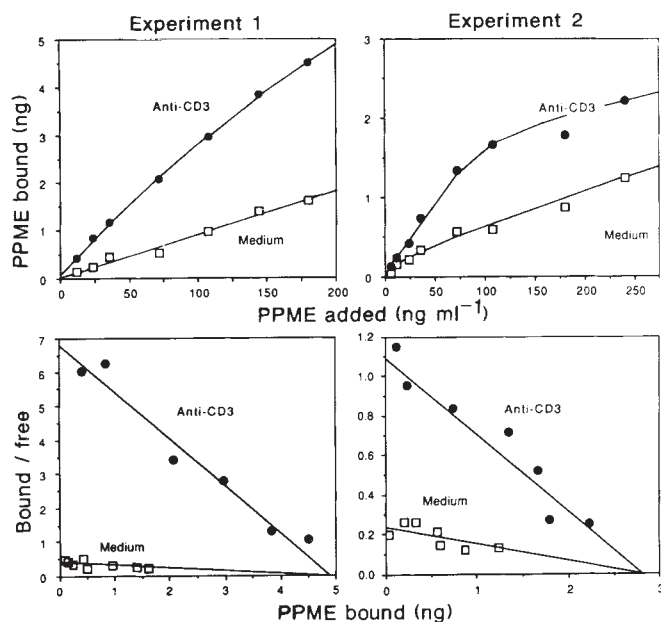


FIG. 3 The affinity of LAM-1 for PPME increases following lymphocyte activation. Lymphocytes were stimulated by crosslinking CD3 at 4 °C for 20 min (Fig. 1) and incubated for 60 min at 4 °C with the indicated amounts of [125 I]-labelled PPME in PBS or PBS containing 5 mM EDTA. After processing as in Table 1, the amount of Ca $^{2+}$ -dependent binding was calculated as a function of the specific radioactivity of the [125 I]PPME added and the maximal binding capacity of this reagent. PPME binding in the presence of EDTA was less than 10% of the values shown and 80–95% of the PPME binding was blocked by anti-LAM-1-3. Values are mean c.p.m. obtained from replicate tubes and s.d. were less than 10%. In a third experiment, lymphocyte stimulation through the CD2 pathway induced a similar increase in LAM-1 affinity for PPME.

stimulating agents at 37 °C (Fig. 1). The increase in receptor activity was also observed at 4 °C, though more slowly. This is consistent with studies of other surface receptors showing that many signal transduction pathways function at 4 °C (Refs 21–24). Crosslinking of CD3 on lymphocytes at 4 °C resulted in an increase in PPME binding that peaked at 15–30 min and returned to basal levels within 1–2 h. Again, cell surface expression of LAM-1 did not change. By contrast, activation of lymphocytes at 37 °C induced a rapid increase in PPME binding which peaked within the first few minutes of activation and quickly diminished (Fig. 2). The rapid decay in PPME binding following CD3 crosslinking at 37 °C most probably results from the transient nature of the activity increase in addition to increased LAM-1 shedding^{10,18}. A maximum increase in PPME binding by neutrophils was observed 5 min after activation at 37 °C (Fig. 1). This increase preceded LAM-1 shedding, which occurred maximally after 30 min¹². The increased binding of PPME at early time points was inhibited by anti-LAM-1-3 or EDTA. Prolonged activation of neutrophils, however, resulted in PPME binding that was not inhibited by antibody or EDTA as described²⁵. Therefore, alterations in LAM-1 ligand binding activity occur rapidly and are most easily studied at 4 °C, where the time course of leukocyte activation is slower and LAM-1 shedding is minimized.

Because PPME functions as a soluble ligand, it was possible to determine whether the increase in PPME binding resulted from an increase in the binding affinity of LAM-1 (Fig. 3). Binding of PPME by LAM-1 was linear at PPME concentrations between 10 and 200 ng ml $^{-1}$ and reached equilibrium after 60 min at 4 °C. Scatchard analysis of these data does not provide a binding constant for LAM-1 because PPME is polyvalent. The difference in LAM-1 binding affinity observed between stimulated and unstimulated lymphocytes was, however, quite striking, varying from 4.5- to 17-fold in three experiments (Fig. 3). Therefore, activation of leukocytes may induce an increase in the affinity of LAM-1 for its ligand(s) which is likely to mediate enhanced leukocyte adhesion.

It has not previously been clear why LAM-1 is expressed by many cell types that do not normally migrate into lymph nodes^{3,12}. The studies reported here suggest that leukocyte migration is likely to be influenced by cell-specific activation signals. Specifically, we predict that cellular activation induces a conformational change in LAM-1 that leads to enhanced receptor affinity. Presumably, the cytokines and other signals which activate neutrophils and thereby upregulate LAM-1 affinity would be concentrated *in vivo* only at sites of inflammation, resulting in localized adhesion. Moreover, the ability to rapidly and transiently increase affinity may be a general property of leukocyte adhesion molecules, because the activity of the CD11/CD18 and VLA integrins is also increased by lymphocyte activation^{26,27}. Thus, leukocyte migration and tissue recognition *in vivo* may involve the sequential activation and deactivation of several adhesion molecules in addition to LAM-1. This is likely because monocytes express LAM-1, yet have distinct patterns of trafficking. Thus, LAM-1 may be principally involved in the initial interactions between leukocytes and endothelium, whereas other adhesion molecules would have a broader role in subsequent adhesion and diapedesis into tissues after LAM-1 shedding¹². Endothelial binding and leukocyte migration through LAM-1 is thus regulated in three ways: first, by expression of the receptor on distinct leukocyte subpopulations; second, as shown here, by an activation-induced transient increase in receptor affinity for ligand; and third, by the subsequent shedding of the receptor from the cell surface. □

Received 10 October; accepted 14 December 1990.

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ACKNOWLEDGEMENTS. We thank S. Rosen, M. Slodki, S. Cannistra and L. Stoolman for providing PMME and suggestions. This work was supported by the NIH, and the Swiss National Foundation for Scientific Research (to O.S.).

Fluorescence ratio imaging of cyclic AMP in single cells

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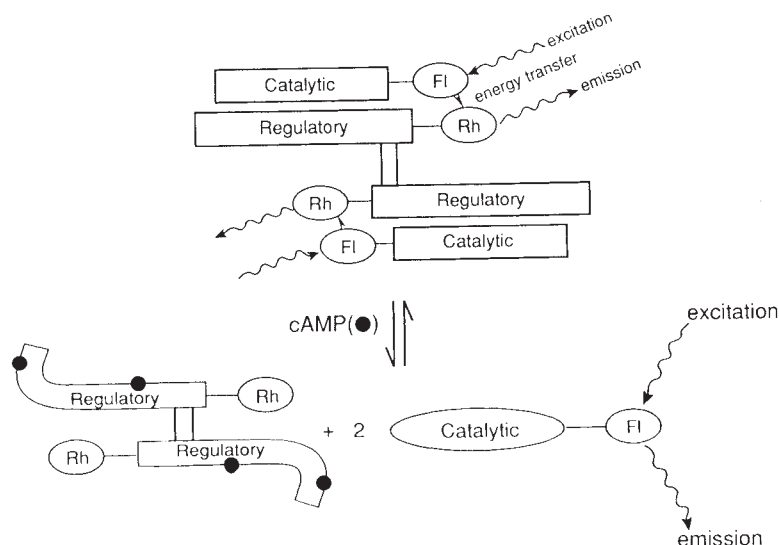
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FLUORESCENCE imaging is perhaps the most powerful technique currently available for continuously observing the dynamic intracellular biochemistry of single living cells¹. However, fluorescent indicator dyes have been available only for simple inorganic ions such as Ca^{2+} , H^+ , Na^+ , K^+ , Mg^{2+} and Cl^- . We now report a fluorescent indicator for the adenosine 3',5'-cyclic monophosphate (cAMP) signalling pathway. The sensor consists of cAMP-dependent protein kinase² in which the catalytic (C) and regulatory (R) subunits are each labelled with a different fluorescent dye such as fluorescein or rhodamine capable of fluorescence resonance energy transfer in the holoenzyme complex R_2C_2 . When cAMP molecules bind to the R subunits, the C subunits dissociate, thereby eliminating energy transfer. The change in shape of the fluorescence emission spectrum allows cAMP concentrations and the

activation of the kinase to be nondestructively visualized in single living cells microinjected with the labelled holoenzyme.

The C_α and R^1 isoforms of the kinase subunits have been cloned and expressed at high levels in *Escherichia coli*^{3,4}. We have now labelled C_α with fluorescein isothiocyanate and R^1 with tetramethylrhodamine isothiocyanate (Fig. 1). After many trials, reaction conditions were found in which one or two dye molecules could be covalently linked to each subunit without affecting recombination into holoenzyme. In the holoenzyme, the dyes are close enough so that excitation of the fluorescein donor at 480–495 nm results in detectable emission from the rhodamine acceptor moiety as a result of resonance energy transfer^{5–7} (Fig. 2a). Cyclic AMP liberates the C subunits, effectively increases the donor–acceptor distance to infinity, and thereby prevents energy transfer. Excitation of the fluorescein now gives brighter emission of that dye at 500–570 nm and less emission at the 570–620 nm wavelengths characteristic of rhodamine. Therefore cAMP changes the ratio of emission amplitudes at two wavelength bands; such ratioing cancels out intensity variations due to probe concentration, optical path length, and excitation intensity, and is highly desirable for microscopic imaging^{1,8}. A proposed abbreviation for the labelled holoenzyme is FICRhR (pronounced 'flicker'). The calibration curve for FICRhR emission ratio versus free cAMP is essentially superimposable upon the curves describing kinase activation for either labelled or native enzyme (Fig. 2b), all being half maximal at about 90 nM cAMP with slightly positive cooperativity. Thus the labelling does not alter the affinity for cAMP or

FIG. 1 Schematic diagram showing how to detect cAMP using fluorescence energy transfer^{5–7,24} between two fluorophores, for example fluorescein and rhodamine, attached to the catalytic (C) and regulatory (R) subunits, respectively, of cAMP-dependent protein kinase. The protein was labelled as follows: recombinant C_α and R^1 subunits^{3,4} (approximately 0.5–2 mg ml⁻¹) of mammalian cAMP-dependent protein kinase, were dialysed separately against 25 mM bicine, 0.1 mM EDTA for 4 h at pH 8.0, 0 °C. The catalytic subunit was labelled with 0.3 mM fluorescein 5'-isothiocyanate in the presence of 8 mM MgCl_2 and 5 mM ATP to prevent inactivation of kinase activity. The regulatory subunit was labelled with 0.5 mM tetramethylrhodamine isothiocyanate (isomer G). Both dye reagents were from Molecular Probes, Eugene, Oregon; labellings were allowed to proceed for 30 min at room temperature, then quenched by addition of 5 mM glycine for 10–15 min. The excess dye was removed by passing each protein solution through a Sephadex G-25 column (3 ml), eluting with 25 mM potassium phosphate pH 6.8, 2 mM EDTA, 5 mM mercaptoethanol and 10% glycerol. The first coloured band was collected. Covalent attachment of the dyes to the proteins was verified by gel electrophoresis under denaturing conditions. The dye:protein stoichiometries were determined by absorbance spectrophotometry to be 1.1 fluoresceins per catalytic subunit and 1.0 tetramethylrhodamines per regulatory subunit monomer, assuming extinction coefficients (in $10^3 \text{ M}^{-1} \text{ cm}^{-1}$) of 65 and 11 for protein-bound fluorescein²⁵ at 495 and 280 nm, 72 and 18 for protein-bound tetramethylrhodamine²⁴ at 552 and 280 nm, 45 for catalytic at 280 nm, and 48 for regulatory at 280 nm. The subunits were then mixed at equal concentrations by weight, typically about 0.5 mg ml⁻¹, and dialysed against 25 mM potassium phosphate pH 6.8, 0.5 mM MgCl_2 , 0.1 mM ATP,



5 mM mercaptoethanol, 5% glycerol for 3–5 days at 4 °C. Formation of holoenzyme was monitored by kinase assay²⁶. A wide variety of other visible-wavelength fluorophores and linking groups (for example N-hydroxy-succinimides, iodoacetamides) have been tested on both isolated subunits and preformed holoenzyme, but the above procedure so far gives the best combination of yield, convenience, ability to reform holoenzyme, and optical sensitivity.

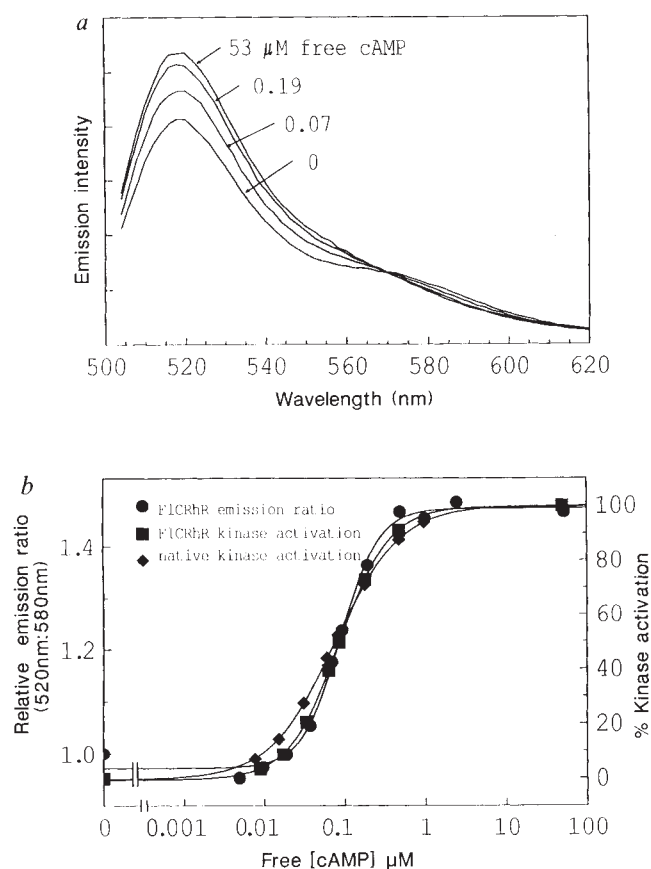


FIG. 2 Responses of FICRrH to cAMP *in vitro*. *a*, Change in fluorescence emission spectrum of FICRrH on titration with cAMP when the fluorescein is excited at 495 nm. The solutions contained 3 nM FICRrH in 130 mM KCl, 5 mM MgCl_2 , 3 mM ATP, 10 mM 3-(N-morpholino)propanesulphonate, pH 7.2, at 22 °C. Excitation and emission bandwidths were 1.8 and 4.6 nm, respectively. For clarity only two intermediate concentrations of cAMP are shown. Full saturation with cAMP increases the emission from fluorescein at 520 nm by about 31% and decreases the emission from rhodamine by about 13%, resulting in a 1.5-fold increase in ratio of 520 nm amplitudes in this batch of FICRrH. The reason why the 520 nm amplitude changes by a larger percentage than the 580 nm is probably because the latter includes not only energy transfer but also spectral overlap, that is 580 nm emission from fluorescein and 490 nm excitation of rhodamine, signals insensitive to cAMP-induced dissociation of the holoenzyme. Control experiments with fluorescein-labelled C paired with unlabelled R showed no significant change in emission spectrum or quantum yield (0.42) on binding cAMP, though the kinase became activated. Therefore the ratio change required labelling of R. Likewise when the rhodamine in FICRrH is directly excited at 540 nm, its emission spectrum and quantum yield are insensitive to cAMP. For this reason, emission ratioing at 520 nm versus ≥ 570 nm while exciting at 490 nm is more sensitive to cAMP (1.5-fold range of ratios) than excitation ratioing at 490 versus 540 nm excitation efficiencies while observing at ≥ 570 nm (1.13-fold range of ratios). The energy transfer efficiency of 0.31 in the holoenzyme corresponds to a distance between the fluorophores of 5.8 nm assuming random orientations for the transition moments. This distance is reasonable for the holoenzyme, as its Stokes radius is 4–5 nm (ref. 27) and the distance between the active sites in the two catalytic subunits exceeds 5 nm (ref. 2). *b*, Ratio of emissions at 520 nm to 580 nm (circles, left-hand scale), normalized to its value at zero cAMP, with the percentage activation of the labelled (squares) and unlabelled (diamonds) kinase (right-hand scale), all as a function of free cAMP. Kinase activity was measured as in ref. 26. Free cAMP was calculated from total cAMP by subtracting a small correction for bound cAMP, assuming that two molecules must bind to each R to release C. Curves were fitted by least-squares to the Hill equation; the concentrations for half-maximal activation were 88, 86 and 76 nM, and the Hill coefficients were 1.8, 1.4 and 1.1 for emission ratio, labelled kinase, and native kinase respectively. The internal differences in these sets of values are probably not significant because they are less than batch-to-batch variation in protein.

the activity of the freed C subunits. Ideally one would like yet higher efficiency of energy transfer in the holoenzyme to increase the overall range of emission ratios. Efforts are continuing in this direction, but FICRrH is already good enough to permit many cell biological applications.

FICRrH has been injected into smooth muscle (BC3H1) and fibroblast (REF-52) cell lines at estimated final concentrations approximating or exceeding intrinsic holoenzyme (0.2–2 μM)⁹. Ratioing of fluorescence emissions at 500–530 nm to ≥ 570 nm and digital image processing reveals the dissociation of FICRrH, which can be related to the intracellular free cAMP concentration using Fig. 2*b*. Figure 3*a, b* shows recordings of the spatially averaged ratio from single BC3H1 cells. Before stimulation, the emission ratio was essentially stable at a level indicating that the FICRrH was remaining intact without dissociation. Treatment of the cells with extracellular dibutyl cAMP resulted in a sigmoidal rise in the fluorescence ratio (Fig. 3*a*). Supramaximal doses of β -adrenergic agonists produced faster increases in cAMP leading to 100% activation within 1 min (Fig. 3*b*). Removal of the agonist and blockage of the β -receptors with 100 nM propranolol caused a slow decrease in indicated cAMP concentration, which could then be increased again by direct activation of adenylyl cyclase with forskolin¹⁰. The reversibility of the emission ratio change was somewhat surprising and gratifying, since we had feared that considerable scrambling of exogenously labelled and native unlabelled subunits might occur. Such scrambling would have prevented reconstitution of energy transfer and detection of falling cAMP levels. In analogous experiments, treatment of FICRrH-injected REF-52 fibroblasts with 10 μM prostaglandin E_1 (ref. 11) gave detectable rises in cAMP levels in 51 of 60 cells, two of which are shown in Fig. 3*c*. 5'-(N-ethylcarboxamido)adenosine, an adenosine analogue, (at 50 μM) gave similar increases in 9 of 10 cells respectively. Again these changes in emission ratio reversed on removal of agonist. The lack of FICRrH response in some cells (see Fig. 3*c*, broken line) was not due to deterioration of probe molecules, as forskolin worked as expected, but may instead reflect genuine cell-cell heterogeneity analogous to that often seen in Ca^{2+} signalling^{12,13}. Heterogeneity is most directly assessed by single-cell assays, and can strongly affect the interpretation of population measurements.

Figure 4 shows pseudocolour images of the emission ratio and subcellular localization of FICRrH in a REF-52 fibroblast. Elevation of cAMP first dissociated FICRrH and increased its emission ratio, whereupon the freed C subunit gradually translocated to the nucleus¹⁴. A new finding was that removal of cAMP stimulation could undo the nuclear localization and reconstitute holoenzyme and fluorescence energy transfer in the cytoplasm. These results were typical of both REF-52 and BC3H1 cells and could be explained by reversible diffusion of C subunits between cytoplasmic R^1 and nuclear binding sites, where R^1 wins the competition when cAMP is low. Also, the continued responsiveness of FICRrH argues that major degradation was not occurring over the first 4 h after injection. It will be interesting to repeat such experiments with other isozymes of R and C, which may behave differently and give hints as to why so many isoforms exist².

A question that will need answering in each application is whether the injected kinase affects the cell physiology, either by buffering cAMP or by increasing its downstream efficacy. Past experience suggests that injection of holoenzyme has little effect whereas unbalanced regulatory or catalytic subunits respectively mimic inhibition or overstimulation of cAMP signalling^{15–17}. This is a probable advantage of basing the sensor on holoenzyme rather than on another cAMP-binding protein such as R subunits alone or the cAMP receptor protein¹⁸ of *E. coli*, which would divert cAMP to nonproductive binding sites. FICRrH inherently covers the most interesting concentration range of cAMP because it has the same affinity for cAMP as the native target enzyme (Fig. 2*a*). Its use will be most

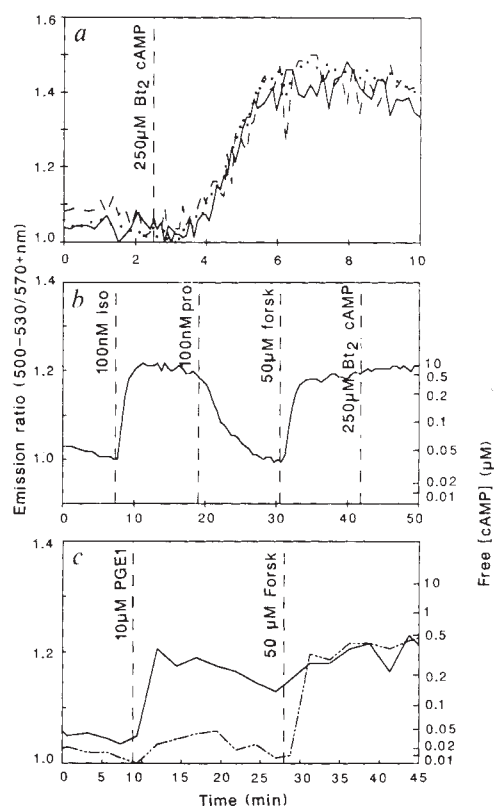
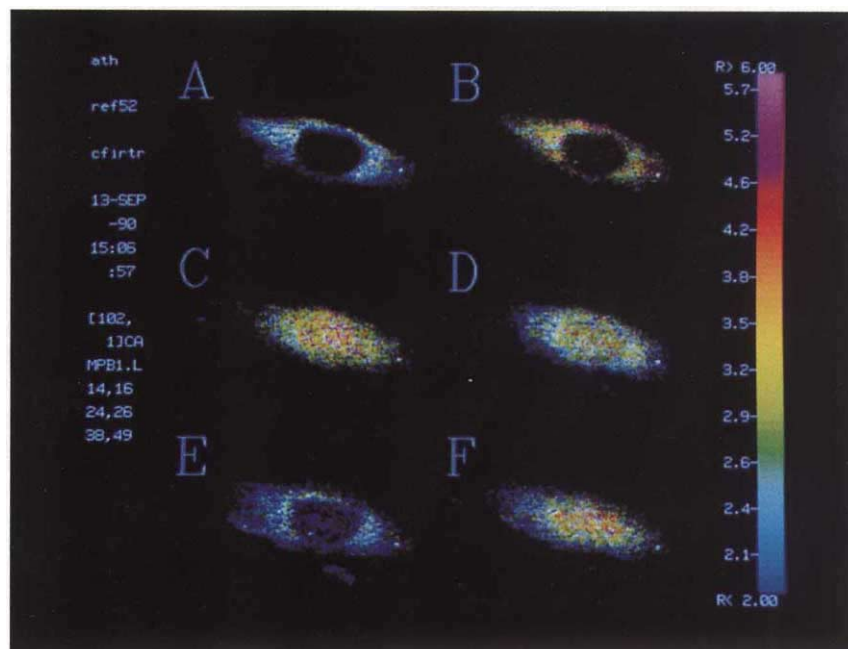


FIG. 3 Time courses of FICRHR emission ratio and cAMP in single cells. *a*, In three BC3H1²⁸ cells injected with FICRHR, 250 μ M dibutyl cAMP (Bt₂cAMP) causes a delayed increase in the ratios of fluorescence emission at 500–530 nm to $\geq$ 570 nm. The three cells were simultaneously monitored by digital imaging microscopy²⁹ and are represented by solid, dashed, and dotted lines, respectively. The initial delay of about 2 min may reflect the time required for the cAMP analogue to cross the membrane and undergo hydrolysis of butyryl groups before becoming active. *b*, FICRHR response is reversible. In a single BC3H1 cell, the emission ratio increases rapidly in response to the β -adrenergic agonist isoproterenol²⁸ (100 nM). Similar increases were detected in every one of 50 cells exposed to 50–100 nM isoproterenol. The emission ratio then decreases when the isoproterenol is

FIG. 4 Pseudocolour images of FICRHR emission ratio and subcellular localization in a single REF-52 fibroblast. Increasing ratios of 500–530 to $\geq$ 570 nm intensities are coded in pseudocolour hues ranging through the spectrum from blue to red, as calibrated in the right hand colour scale. Pseudocolour brightness reflects the mean of the two emission intensities. *a*, Before stimulation, the cytoplasm shows a low ratio (blue), whereas the nucleus appears black because the holoenzyme is excluded. *b*, 5.5 min after addition of 2 μ M prostaglandin E₁ (PGE₁), the emission ratio has risen (yellow pseudocolour) showing an increase in cAMP, but the nucleus is still mostly dark because it is only just beginning to take up catalytic subunit. *c*, 40.5 min after addition of PGE₁, the nucleus now contains a considerable amount of catalytic subunit, which by itself gives a higher ratio (redder pseudocolour) than when it is intermingled with rhodamine-labelled regulatory subunit. *d*, 7 min after removal of PGE₁ from the medium, emission ratios and cAMP levels are declining, though exit of catalytic subunit from the nucleus is far from complete. *e*, 66 min after removal of PGE₁, emission ratios (blue) and cAMP levels have recovered to baseline levels, and the nucleus has been cleared of catalytic subunit. *f*, 45 min after addition of 50 μ M forskolin to activate adenylyl cyclase, emission ratios and cAMP are high and the nucleus has filled in again. The total elapsed time from injection of FICRHR was 4 h at this point. The absolute range of ratios indicated by the right-hand colour scale differs from those in Fig. 3 because the present values have not been normalized against their lowest value. These results were obtained at 22 °C; translocation of the catalytic subunit is faster at 37 °C.



replaced by the β -antagonist propranolol (100 nM), and finally increases again when 50 μ M forskolin is added to stimulate adenylyl cyclase directly¹⁰. The FICRHR is then fully saturated with cAMP, as Bt₂cAMP has no further effect. The right-hand ordinate axis represents an attempt to calibrate the ratio changes in terms of free cAMP concentrations. Ratios measured on the microscope are measured with a television camera and filters defining relatively broad bandwidths to maximize detection efficiency, so they differ quantitatively from those measured in a spectrofluorometer cuvet with a photomultiplier and monochromators with narrow bandwidth. This experiment was calibrated by assuming that the mean ratio obtained in parallel runs by coinjecting FICRHR with the cAMP antagonist *R*_p-adenosine cyclic 3',5'-phosphorothioate³⁰ (BioLog Life Science Institute, La Jolla, California; 1 mM pipette concentration) represents zero cAMP, whereas the mean ratio obtained after 250 μ M Bt₂cAMP corresponds to saturating cAMP. The Hill coefficient and cAMP concentration for half-maximal activation were taken from *in vitro* measurements on the same batch of FICRHR. *c*, Two adjacent REF-52 fibroblasts²⁹ (solid and dashed lines) respond differently to 10 μ M prostaglandin E₁ (PGE₁). The cell represented by the dashed line subsequently elevates cAMP when challenged with 50 μ M forskolin, showing that its adenylyl cyclase and FICRHR were functional. The cAMP calibration was calculated by a different protocol from *b*, in which the lowest ratio observed before stimulation was assumed to represent zero cAMP. The ratio representing saturating cAMP was assumed to be 1.33-fold higher, because when a parallel aliquot of this batch of FICRHR was sandwiched as a thin film between coverslips with 0 or 1 mM added cAMP, the emission ratios differed by a factor of 1.33. The two calibration procedures in *b* and *c* both have advantages and disadvantages and probably over- and underestimate free cAMP, respectively. FICRHR in REF-52 cells showed no response to 5% serum or 2 μ M phorbol 12,13-dibutyrate (data not shown), indicating independence from other signalling pathways such as receptor tyrosine kinase, inositol phospholipid turnover, cytosolic Ca²⁺ spikes, and protein kinase C. METHODS. Injection and imaging. BC3H1 and REF-52 cells in log phase growth were pressure-injected with 7.6–50 μ M FICRHR, 25 mM potassium phosphate pH 7.36, 1 mM EDTA, 1 mM mercaptoethanol, and 5% glycerol. Injection volumes were 2–10% of cell volume. Digital images of the fluorescence were obtained on a system previously described²⁹, except that the excitation wavelength was fixed at 490 nm (4.6 nm bandpass) and two emission bands were sequentially sampled by alternately placing a 500–530 nm bandpass interference filter (Karl Feuer Optical Associates, Montclair NJ) and a 570 nm long-pass filter (EF570LP, Omega Optical, Brattleboro, Vermont) in front of the silicon intensified target camera (Dage-MTI, Michigan City, Indiana). Ratios are averages over the entire cell. Because of residual spatial non-uniformities in the filters, absolute ratios varied somewhat from one part of the field of view to another, so for mutual comparability the ratios for each cell have been normalized against the lowest ratio seen for that cell during the experiment. All data shown were obtained at 22–23 °C.

advantageous when cells are scarce or heterogeneous, when high spatial and temporal resolution are required, or when cAMP or its kinase may be bound or compartmentalized so that traditional destructive measurements of total cAMP or activity ratio are inadequate¹⁹⁻²¹. Fluorescence resonance energy transfer is already known to be biologically useful in detecting colocalization of membrane components⁵⁻⁷ and hybridization of nucleic acids²²; one can now imagine further applications in intracellular signalling, such as detecting cyclic GMP using mutated²³ cAMP-dependent kinase, or analysing interactions between subunits of GTP-binding proteins, between calmodulin and its target enzymes, or between components of transcription-regulating complexes. □

Received 9 October; accepted 20 December 1990.

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ACKNOWLEDGEMENTS. This work was supported by the NIH and HHMI.

Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb

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THE synapsins are a family of closely related phosphoproteins (termed synapsins Ia, Ib, IIa and IIb) associated with synaptic vesicles and implicated in the short-term regulation of neurotransmitter release from nerve endings¹⁻⁵. During development, expression of the synapsins correlates temporally with synapse formation⁶, but there has been no direct evidence that they are involved in synaptogenesis. Here we report that overexpression of synapsin IIb in the neuroblastoma x glioma hybrid clonal cell line NG108-15 leads, during cell differentiation, to marked increases in the number of neuritic varicosities and in the numbers of small clear vesicles and large dense core vesicles per varicosity, as well

as to the appearance of synapse-like cell-cell contacts. Thus, synapsin IIb may be involved in the regulation of synapse formation and, as a result, in long-term neuronal signalling.

To explore the role of the synapsins in synaptogenesis⁶, we overexpressed synapsin IIb because it is the least complex and the shortest of the four synapsins. Domains A, B and C of synapsin IIb, which together make up 88% of the total synapsin IIb molecule, exhibit a high degree of homology with the other three known isoforms of synapsin⁵. A mammalian expression vector that contained a complementary DNA insert encoding the complete sequence of rat synapsin IIb⁵ (pcDNA-IIb) was transfected into NG108-15 cells which have a low endogenous level of synapsin IIb (data not shown). The NG108-15 cell line, on induction of cell differentiation using agents that raise intracellular cyclic AMP, expresses virtually all important neuronal properties previously found for cultured primary neurons⁷⁻⁹. In particular, differentiated NG108-15 cells can form functional synapses with cultured myotubes^{7,8}.

We analysed the morphology of differentiated NG108-15 cells that had been transfected with vector containing synapsin IIb cDNA ('NG108-IIb cells') so as to overexpress synapsin IIb. The phenotype of these cells differed from that of differentiated cells mock-transfected with vector only ('NG108-mock cells'). Differentiated NG108-IIb cells had many more varicosities (3-10 µm in diameter) along their neurites than did differentiated NG108-mock cells (Fig. 1a,b). The difference became evident 3 days after induction of differentiation and persisted thereafter. Three days after the induction of differentiation the average number of varicosities per cell (Fig. 1c) and per neurite (data not shown) for eight NG108-IIb clones was three times that for eight NG108-mock clones. A similar difference in the number of varicosities per cell was present at 7 days of differentiation (data not shown), when all cells had responded to the differentiating conditions by extending neuritic processes (see ref. 7). The differentiated NG108-IIb cells also had substantially more varicosity-neurite and varicosity-cell-body contacts than differentiated NG108-mock cells cultured under the same conditions (Fig. 1a,b).

Ultrastructural studies revealed that the varicosities of differentiated NG108-IIb cells contained more small clear vesicles (40-70 nm in diameter), large dense-core vesicles (80-160 nm in diameter), bundles of cytoskeletal elements, coated vesicles, multivesicular bodies, mitochondria, and membranous structures similar to smooth endoplasmic reticulum than did the varicosities of differentiated NG108-mock cells (Fig. 1d-f). Counts of vesicles per varicosity in randomly chosen varicosities from 10 NG108-mock and 10 NG108-IIb cells revealed that NG108-IIb cells had 12 times more small clear vesicles and six times more large dense core vesicles. (That the NG108-mock cells had few small clear vesicles 3 days after differentiation is in agreement with results for nontransfected NG108-15 cells⁹.) The varicosities of differentiated NG108-IIb cells resemble those typically seen in neurons of the autonomic nervous system, in which small clear vesicles and large dense-core vesicles are colocalized¹⁰. In addition, many synapse-like contacts characterized by synaptic membrane specializations were found in cultures of NG108-IIb cells. These synapse-like contacts involved either a varicosity and a neuritic process (Fig. 1g) or a varicosity and a cell body. No such synapse-like specializations were found between NG108-mock cells.

Southern blot analysis using genomic DNA prepared from individual NG108-IIb and NG108-mock cell lines confirmed that, after transfection and selection, the introduced rat synapsin IIb cDNA was stably incorporated into the genome of the NG108-IIb cell lines (Fig. 2A). Immunoblot analysis of protein extracts showed that the undifferentiated NG108-IIb cells (log-phase growth) expressed seven times more synapsin IIb than did NG108-mock cells (Fig. 2Ba, Table 1). Three days after differentiation, the level of synapsin IIb in NG108-IIb cells was 24 times that in NG108-mock cells (Fig. 2Bb; Table 1). Results

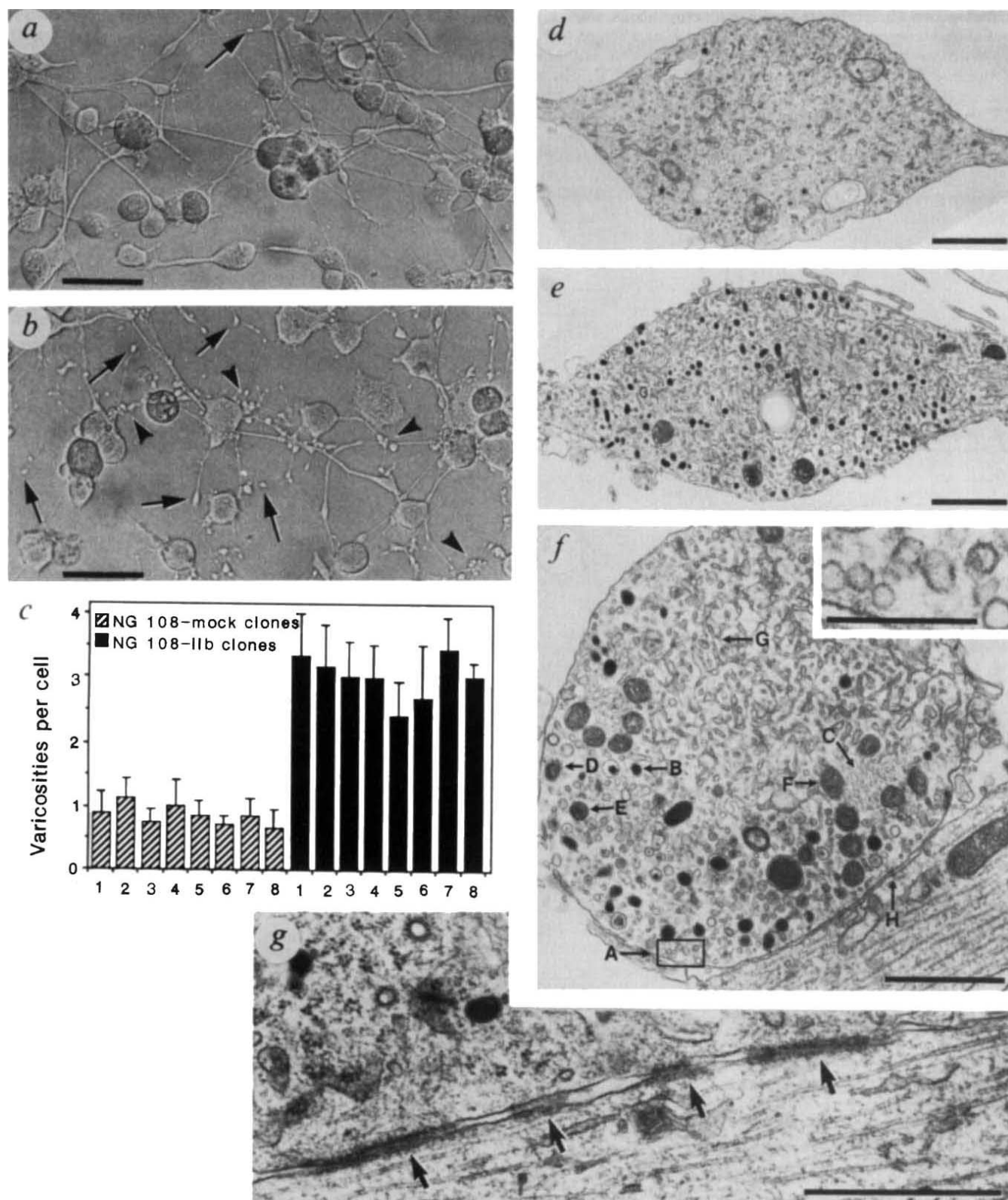


FIG. 1 Effect of transfection with synapsin IIb on morphology of NG108-15 cells. *a* and *b*, Representative light photomicrographs (Nomarski optics) of differentiated cells which had been transfected with vector only (NG108-mock cells) (*a*) or with vector containing synapsin IIb cDNA (NG108-IIb cells) (*b*). Note the increased number of varicosities (arrows) and also the appearance of varicosities contacting neurites and cell bodies (arrowheads) in cultures of NG108-IIb. Bars, 100 μ m. (*c*) Quantitative analysis of the number of neuritic varicosities in eight mock-transfected clonal cell lines (NG108-mock1-8) and eight synapsin IIb-transfected clonal cell lines (NG108-IIb1-8). Values are expressed as the number of varicosities per cell and represent

means $\pm$ s.d. ($n=12$). Grouped average of data from the eight individual NG108-IIb clones is significantly different from grouped average of data from the eight individual NG108-mock clones using a one-way analysis-of-variance test followed by a Tuckey test ($P<0.001$). A similar fold-increase on transfection with synapsin IIb was obtained when results were calculated as varicosities per neurite. *d-g*, Electron micrographs of NG108-mock and NG108-IIb cells, each differentiated for 3 days (bars, 1 μ m). Representative profiles of neuritic varicosities in NG108-mock2 (*d*) and NG108-IIb2 (*e*) cells are shown. An example of a varicosity making contact with a neurite (*f*) in a culture of NG108-IIb cells is shown; arrows point to a cluster of small

clear vesicles (A), a large dense core vesicle (B), a bundle of cytoskeletal elements (C), a coated vesicle (D), a multivesicular body (E), a mitochondrion (F), structures similar to smooth endoplasmic reticulum (G) and a synapse-like contact (H). Enlargement of the cluster of small clear vesicles (A) in the varicosity is shown in the inset (Bar, 0.25 μm). *g*, Representative photomicrograph showing a junctional complex (arrows) between a varicosity and a neurite in NG108-IIb cells.

METHODS. Cell culture. Cells were grown in monolayer cultures⁹ in dishes coated with human placental collagen (Sigma)¹⁷, using DMEM medium supplemented with 10% FBS, 0.1 mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine (HAT) and were maintained at 37 °C in a humidified incubator equilibrated with 95% air and 5% CO₂. To induce neuronal differentiation, cells were treated with differentiating medium (10 μM prostaglandin E1 and 1 mM isobutyl methylxanthine in DMEM supplemented with 5% horse serum and HAT). Transfection: Rat synapsin IIb cDNA⁵ was inserted into the mammalian expression vector pcDNA1 (Invitrogen) under control of a cytomegaloviral promoter using EcoRI and BamHI restriction enzyme cleavage sites. The synapsin IIb expression construct pcDNA-IIb (10 μg) and the neomycin resistance vector pMAM-neo (10 μg ; Clontech) were co-transfected into undifferentiated (logarithmic growth) NG108-15 cells using the calcium phosphate precipitation method¹⁸. Mock cotransfection of the pcDNA1 (without insert) and pMAM-neo vectors served as control. Multiple dishes were transfected. Stable neomycin-resistant clones were isolated by ring cloning after three weeks of selection in the presence of G418 (0.8 mg ml⁻¹) (Geneticin; Gibco). To avoid subclones generated by cell migration, only one clone per tissue culture dish was used for the analysis. Transfected clonal cell lines were passaged 25 times before being used in this study. Quantitation of numbers of varicosities. Eight individual synapsin IIb-transfected clonal cell lines (NG108-IIb1–8) and eight individual mock-transfected control clonal cell lines (NG108-mock1–8) were each plated at equal density (2×10^5 cells per 100-mm dish) on separate collagen-coated dishes. Cells were treated with differentiating medium to induce neuronal differentiation. The number of neurites per cell and the average length of the neurites were not significantly different between NG108-mock and NG108-IIb cells at any time after induction of differentiation (data not shown). Three days after the induction of differentiation, the number of varicosities per cell for each clonal cell line was determined by using a light microscope at 250 magnification (Leitz, Labovet) under blind conditions. Multiple random views were chosen for analysis. A total of 1,520 NG108-mock cells and of 1480 NG108-IIb cells were counted. Electron microscopy. Two NG108-mock cell lines (NG108-mock2 and 3) and two NG108-IIb cell lines (NG108-IIb 2 and 3) were grown in collagen coated dishes and differentiated for 3 days under identical culture conditions. Cells were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After washing with buffer alone, the cells were postfixed in 1% OsO₄ in 0.1 M cacodylate buffer, stained with uranyl acetate, dehydrated in ethanol and embedded in Epon. After polymerization of the Epon, the culture plates were examined by light microscopy, and selected areas were cut out and glued to specimen blocks. Cells were sectioned parallel to the culture plate substrate. Sections were stained with uranyl acetate and lead citrate, and examined using a Jeol 100CX electron microscope.

similar to those shown in Table 1 for synapsin IIb were observed in each of three experiments. Immunoblot analysis using antibodies specific for either dephosphorylated synapsin IIb or phosphorylated synapsin IIb (ref. 11) showed that, as for endogenous synapsin IIb in NG108-mock cells, the overexpressed synapsin IIb in NG108-IIb cells was partially phosphorylated, indicating that the introduced synapsin IIb can be phosphorylated by endogenous protein kinase activity (data not shown).

Levels of the other three synapsins and of synaptophysin, an integral membrane protein of synaptic vesicles¹², in NG108-IIb cells were substantially higher than those in NG108-mock cells. The increases in the amounts of these synaptic vesicle proteins were even more dramatic 3 days after induction of differentiation (Table 1). In contrast, levels of three other proteins present in, but not specific to, nerve terminals—actin, spectrin and choline acetyltransferase (ChAT)—were unchanged (Table 1). NG108-mock cells were similar to nontransfected NG108-15 cells with respect to the levels of the synaptic vesicle proteins (Table 1), their localization, and their cell morphology before and after differentiation (data not shown).

Double immunofluorescence staining with affinity-purified antibodies showed that synapsin IIb and synaptophysin are concentrated in the Golgi area of undifferentiated NG108-IIb cells (data not shown). After differentiation, these two synaptic vesicle proteins are enriched in and colocalized to neuritic varicosities as well as in the Golgi area (Fig. 3a–c). Consistent with the increased number of varicosities and with the increased expression of synaptic vesicle phosphoproteins, differentiated NG108-IIb cells (Fig. 3a–c) showed stronger staining for synapsin IIb and for synaptophysin, and a higher number of stained varicosities than did differentiated NG108-mock cells (Fig. 3d–f). Similar results were obtained for synapsins Ia, Ib and IIa (data not shown).

The mechanism by which transfection of NG108-15 cells with synapsin IIb enhances synaptogenesis remains to be determined. Several types of experiments may contribute to elucidating this mechanism. These include determining: (1) the fine structural localization of transfected synapsin IIb; (2) the mechanism by which synapsin IIb alters the levels of other synaptic vesicle proteins (for example, transcriptional versus post-transcriptional, increased protein synthesis versus decreased protein breakdown); (3) comparative effects of transfection of synapsin IIb and other synaptic vesicle proteins on synaptogenesis in various cell types; and (4) the physiological properties of the induced varicosities, that is, whether the morphological evidence

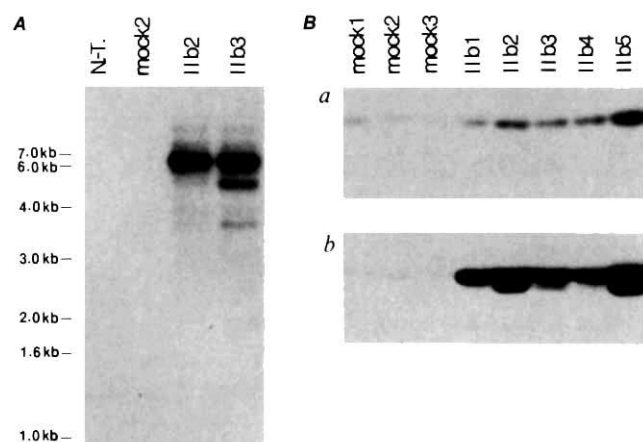


FIG. 2 Southern and immunoblot analysis of NG108-IIb clonal cell lines. *A*, Southern blot analysis using genomic DNA prepared from nontransfected (N-T), NG108-15 cells (lane 1), NG108-mock2 cells (lane 2), NG108-IIb2 cells (lane 3) and NG108-IIb3 cells (lane 4) revealed that the vector containing rat synapsin IIb cDNA had been stably incorporated into the genome of the NG108-IIb cells. *B*, Immunoblot analysis of synapsin IIb in three individual NG108-mock cell lines (lanes 1–3) and five individual NG108-IIb cell lines (lanes 4–8), before (*a*) and after (*b*) differentiation.

METHODS. For Southern blotting, genomic DNA from undifferentiated cells was isolated from nontransfected NG108-15 cells, and from NG108-mock2, NG108-IIb2 and NG108-IIb3 cells (passaged 30 times after transfection), using standard protocols¹⁸ and digested with EcoRI. Digested genomic DNA (10 μg) was fractionated by electrophoresis on a 1.0% agarose gel (along with size markers) and transferred onto a Gene Screen Plus membrane (NEN)¹⁸. The membrane was hybridized for 18 h at 65 °C to rat synapsin IIb cDNA, labelled by random priming (10^9 d.p.m. per μg DNA, 5 ng ml⁻¹), in hybridization buffer containing 1% sodium dodecylsulphate (SDS), 1 M NaCl and 10% dextran sulphate, washed to a final stringency of $0.1 \times \text{SSC}$ and 0.1% SDS at 65 °C, and exposed to X-ray film. For immunoblot analysis, undifferentiated cells, and cells which had been differentiated for 3 days, were solubilized using 1% SDS. After sonication, protein concentration was determined for each SDS extract¹⁹. Equal amounts of protein (200 μg) were run on SDS-PAGE²⁰ and then blotted onto nitrocellulose membranes. The membrane blots were incubated with an affinity purified synapsin IIb antibody followed by ¹²⁵I-labelled protein A and then autoradiographed.

TABLE 1 Effect of expression of synapsin IIb on levels of synaptic-vesicle phosphoproteins and control proteins

	Undifferentiated cells			Differentiated cells		
	NG108-15	NG108-mock (n=4)	NG108-IIb (n=8)	NG108-15	NG108-mock (n=4)	NG108-IIb (n=8)
Synapsin IIb	94	92 ± 25	614 ± 162	282	224 ± 40	5,410 ± 1,214
Synapsin IIa	998	1,090 ± 170	2,060 ± 465	1,724	2,110 ± 185	9,280 ± 1,800
Synapsin I	201	177 ± 49	382 ± 86	399	349 ± 82	2,590 ± 1,100
Synaptophysin	3,305	3,600 ± 480	5,910 ± 765	8,850	10,400 ± 1,100	21,100 ± 2,050
Actin	6,038	5,850 ± 481	5,740 ± 381	6,130	5,980 ± 464	5,860 ± 415
Spectrin	88	105 ± 18	96 ± 14	105	98 ± 19	97 ± 12
ChAT	894	879 ± 110	881 ± 126	937	1,020 ± 125	1,040 ± 161

The levels of each protein were determined in NG108-15 cells, in NG108-mock cells (four clonal cell lines), and in NG108-IIb cells (eight clonal cell lines), before and after differentiation, by immunoblot analysis, as described in the legend to Fig. 2. The values (mean ± s.d.), based on ^{125}I radioactivity, are expressed as arbitrary units, and were calculated from standard curves constructed for each protein.

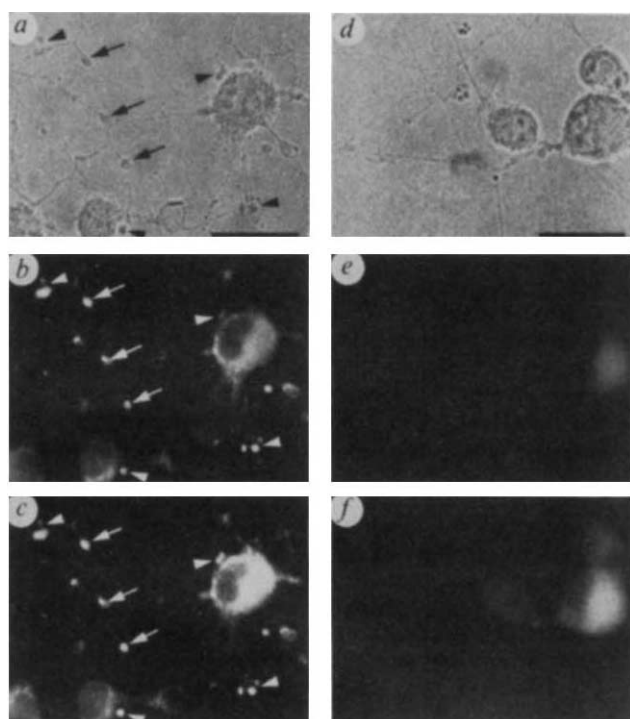


FIG. 3 Double immunofluorescence staining of synapsin IIb and synaptophysin in differentiated NG108-IIb cells (a-c, same field) and differentiated NG108-mock cells (d-f, same field). a,d, Phase-contrast photomicrographs of differentiated cells; b,e, Epifluorescence photomicrographs of cells stained with rabbit anti-synapsin IIb affinity-purified antiserum and fluorescein-conjugated goat anti-rabbit secondary antibody. c,f, Epifluorescence photomicrographs of cells stained with mouse anti-synaptophysin monoclonal antibody and rhodamine-conjugated sheep anti-mouse secondary antibody. Arrows, varicosities; Arrowheads, varicosities contacting neurites or cell bodies. Bars, 100 μm.

METHODS. NG108-IIb cells and NG108-mock cells were plated in collagen-coated dishes (60 mm) at a density of 5×10^5 cells per 60-mm dish. Three hours after plating, the normal medium was replaced with differentiating medium (see legend to Fig. 1) to induce neuronal differentiation. Cells cultured under differentiating conditions for 3 days were washed twice with phosphate buffered saline (PBS, pH 7.4), and then fixed with 3% paraformaldehyde in PBS. The fixed cells were quenched for 20 min in PBS, 0.1 M glycine, and then incubated in PBS, 0.05% Triton X-100 for 5 min to permeabilize the cell membrane. Cells were incubated for 1 h at room temperature with PBS, 10% goat serum and properly diluted primary antibodies. The cells were washed five times in PBS and then incubated with PBS, 10% goat serum and properly diluted rhodamine or fluorescein-conjugated secondary antibodies (Chemicon) for 1 h at room temperature. Cells were washed five times with PBS, covered with coverslips and viewed under a Zeiss fluorescence microscope. Pictures were taken using TMax400 film (Kodak).

for synapse formation is corroborated by electrophysiological evidence.

Neurotransmitters regulate not only the state of phosphorylation of, but also the total amount of, synapsin I in nervous tissue^{6,13-15}. Previous studies have indicated that regulation of the phosphorylation of synapsin I by calcium/calmodulin-dependent protein kinase II is involved in acute regulation of neurotransmitter release^{1-3,16}. The present studies indicate that regulation of synapsin expression could be involved in chronic changes in neuronal signalling. Thus, the synapsins may contribute to both short-term and long-term synaptic plasticity. □

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ACKNOWLEDGEMENTS. We thank M. Nirenberg for the NG108-15 cells, A. Czernik for dephospho- and phospho-specific antibodies against synapsin IIb, and E. Spichas for assistance with electron microscopy. We also thank F. Benfenati and P. DeCamilli for helpful discussion. This research was supported by the National Institute of Mental Health.

Alteration of ionic selectivity of a K^+ channel by mutation of the H5 region

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THE high ionic selectivity of K^+ channels is a unifying feature of this diverse class of membrane proteins. Though K^+ channels differ widely in regulation and kinetics, physiological studies have suggested a common structure: a single file pore containing multiple

Received 23 October; accepted 29 November 1990.

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TABLE 1 Single-channel slope conductances and macroscopic kinetic properties of wild-type and mutants

H5 sequence	Inward slope conductance (pS)			Macroscopic kinetic properties		
	K ⁺ _{ex}	Rb ⁺ _{ex}	NH ₄ ⁺ _{ex}	G _{1/2} (mV)	τ_{inact} (ms)	$h_{1/2}$ (mV)
Wild type	27.2 ± 1.9 (6)	8.7 ± 3.5 (5)	8.3 ± 1.3 (2)	-3.5 ± 8 (2)	10 ± 2 (2)	-35 ± 8 (6)
T441S	24.5 ± 3.6 (3)	16.5 ± 1.8 (4)	38.6 ± 1.6 (2)	-5.4 ± 5 (7)	9 ± 2 (3)	-38 ± 2 (2)
T442S	27.4 ± 3.7 (4)	20.7 ± 2.3 (3)	21.5 ± 3.5 (2)	-24.0 ± 4 (2)	1300 ± 400 (3)	-57 ± 4 (2)
F433Y	20.2 ± 1.1 (4)	20.3 ± 6.2 (2)	11.9 ± 1.8 (2)	5.3 ± 3 (2)	9 ± 6 (3)	-34 ± 9 (7)
F433S	26.4 ± 1.8 (5)	21.7 ± 1.1 (4)	27.0 ± 5.3 (4)	10.0 ± 1 (2)	14 ± 2 (2)	-32 ± 2 (2)

Data are given as mean ± s.d. (n = number of patches). Slope conductances were calculated for the voltage range of -100 to -40 mV from single channel records in bi-ionic conditions with K⁺ internally, and the indicated test ion (K⁺, Rb⁺ or NH₄⁺) externally. Macroscopic kinetic data were derived from two-electrode voltage clamp recordings with 140 mM Na⁺ bath saline. $G_{1/2}$ is the conductance midpoint, indicating the voltages at which the peak conductance was half-maximal, determined from plots of conductance (G/G_0) as a function of voltage, and assuming a reversal potential of -90 mV. τ_{inact} is the time constant of the exponential fit of the first component of inactivation at +45 mV; inactivation was fit simultaneously with two exponentials except for T442S for which a single component was sufficient ($R \geq 0.95$); $h_{1/2}$ is the midpoint of prepulse inactivation, determined from the plot of peak current (I/I_0) as a function of prepulse holding potential.

ion-binding sites and having broader vestibules at both ends¹⁻⁵. We have used site-directed mutagenesis and single-channel recordings to identify a molecular region that influences ionic selectivity in a cloned A-type K⁺ channel from *Drosophila*. Single amino-acid substitutions in H5, the fifth hydrophobic region⁶, enhanced the passage of NH₄⁺ and Rb⁺, ions with diameters larger than K⁺, without compromising the ability of the channel to exclude the smaller cation, Na⁺. The mutations that substantially altered selectivity had little effect on the gating properties of the channel. We conclude that the H5 region is likely to line the pore of the K⁺ channel.

ShB, an alternatively spliced product of the *Shaker* locus in *D. melanogaster*⁷, encodes a transient voltage-dependent K⁺ channel⁸⁻¹¹. The H5 region of this channel is often depicted as an extracellular loop, but its highly conserved sequence and partially hydrophobic nature (Fig. 1) contrast markedly with other putative extracellular loops. These observations support an alternative model in which H5 dips into the plane of the membrane¹². The H5 sequence is not present in Na⁺ or Ca²⁺ channels, but has counterparts in equivalent positions (in the S5-S6 linkers) in each pseudosubunit. There is only limited resemblance between H5 and the S5-S6 linkers, but within each ion channel class, these sequences are highly conserved¹³. Among K⁺ channels, H5 is the region with the highest degree of sequence identity. The intermediate hydrophobicity and conserved nature of H5 suggests that it might contribute to the formation of the pore in K⁺ channels. We tested this hypothesis by characterizing the selectivity and conductance of the wild-type *ShB* channel and examining the effects of single amino-acid substitutions in the H5 region. Many mutations did not yield currents detectable by two-electrode voltage clamp (Fig. 1 legend). Functional channels were obtained when phenylalanine 433 was changed to tyrosine (mutation F433Y; single-letter amino-acid code) or serine (F433S), or when threonines at 441 or 442 were changed to serines (T441S, T442S). Ionic selectivity and conductances of these channels were examined by the patch-clamp technique.

Wild-type currents were recorded in bi-ionic conditions with K⁺ as the internal cation and the test ion NH₄⁺, Rb⁺, K⁺ or Na⁺ as the sole external monovalent cation (Fig. 2a). Bi-ionic recordings provide a direct measurement of cation permeability relative to K⁺ from the reversal potential¹⁴. Permeability was calculated from the reversal potential using a simple rearrangement of the bi-ionic equation from Hille¹⁴:

$$P_X/P_K = \exp[(F/RT)(E_R)]$$

where P_X/P_K is the permeability to the test ion X⁺ relative to K⁺, and E_R is the reversal potential. Calculated permeability ratios (P_X/P_K) for the wild-type *ShB* yielded the sequence K(1.0) > Rb(0.55) > NH₄(0.11) > Na(0.03). This sequence is similar to those of other K⁺ channels^{4,14,15}. Because no inward currents were detected with external Na⁺ at potentials as low as -160 mV, our calculation for P_{Na}/P_K is likely to be an

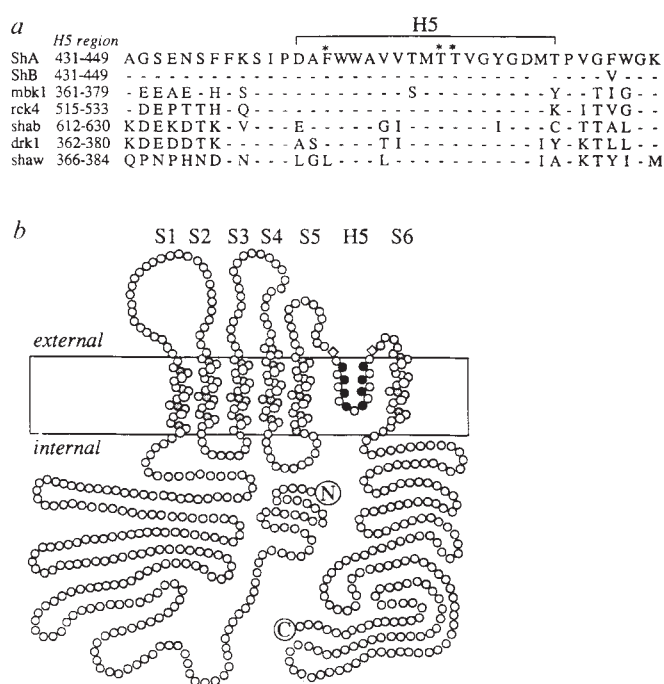


FIG. 1 Amino-acid sequences of the H5 regions of different K⁺ channels, and a schematic model of the proposed transmembrane topology. *a*, Amino-acid sequences of H5 and its flanking regions are compared for various K⁺ channels: ShA⁶, ShB⁷, mbk1²¹, rck4²², shab and shaw²³, and drk1²⁴. Amino-acid numbers corresponding to H5 are at the left. Residues identical to the *Shaker* reference sequence are noted as dashes. The bar (above) marks the H5 region; asterisks indicate the positions of mutations in *ShB* that were studied. Other mutations which did not yield measureable currents with two-electrode voltage clamp and were not studied further were P430G, W434S, W435S, T439A, T439S, T442A, and P450G. *b*, A possible arrangement of the transmembrane spanning sequences of the *ShB* polypeptide, based on hydrophobicity analysis. The H5 region is depicted as tucked into the plane of the membrane as a β-hairpin (alternate residues are filled circles). D431 and T449 (diamonds) contribute to the external vestibule⁵. METHODS. The *ShB* complementary DNA⁶ in Bluescript KS (Stratagene) was transformed into dut⁻, ung⁻ *Escherichia coli* (RZ1032) to provide a single-stranded template for site-directed mutagenesis²⁵. Oligonucleotides (20–30 bases) carrying 1–2 base mismatches at the selected site served as primers for *in vitro* second-strand synthesis by Sequenase DNA polymerase. After transformation in *E. coli* strain BSJ72, mutant colonies were selected by screening with an end-labelled oligonucleotide. To remove possible second site mutations, a cassette region (248 base pairs) containing all H5 and some flanking sequence was excised using restriction sites for *BsmI* and *DraIII*, gel-purified and ligated into a *ShB* clone from which the equivalent H5 cassette had been removed. Cassette regions were then resequenced entirely. The *ShB* clone carried a substitution of the 5' untranslated region with that of *Xenopus* β-globin (provided by W. Kimmerley, UC, San Francisco) to enhance subsequent expression of the messenger RNA in oocytes.

overestimate; the actual reversal potential for Na^+ probably is more negative than our value extrapolated from a linear fit of the outward currents.

Mutations T441S and F433S, which decreased the size and hydrophobicity of residues in H5, increased NH_4^+ permeability (Fig. 2b). Mutation F433Y was more conservative than F433S and produced less change. T442S also had relatively little effect on selectivity. The ratios of $P_{\text{NH}_4}/P_{\text{K}}$ were used to rank mutants and wild type in order of decreasing permeability to NH_4^+ : F433S(1.3) > T441S(0.85) > F433Y(0.28) > T442S(0.21) > wild-type (0.11).

Under identical ionic conditions, the mutations produced differences in inward NH_4^+ conductance. Chord conductances were used for comparison of inward currents for wild-type and mutants (Table 1). Generally, larger inward conductances correlated with increased ionic permeability to the external cation. Inward conductances measured with NH_4^+ were larger than would be expected if selectivity and conductance were directly proportional. For other K^+ channels, similar observations have been explained by proposing multiple binding sites in the pore^{2,14,16}.

The same mutations that were most effective at increasing

permeability to NH_4^+ also increased Rb^+ currents (Fig. 2c). The larger inward currents clearly reflected increased conductances (Table 1) and suggested small positive shifts in reversal potentials. Although the determinations of actual reversal potentials were limited by the uncertainty of the interpolations, that for F433S in particular appeared shifted to the right ($P_{\text{Rb}}/P_{\text{K}} = 1.2$).

Inward conductances in symmetrical K^+ were not affected by the mutations in H5 (Table 1) and, as expected, no differences between wild type and mutants were apparent in the reversal potentials (Fig. 2d). In contrast, mutations at positions which flank both ends of H5 (threonine 449 and aspartate 431) decreased the inward rectification⁵. Wild-type and mutant channels also were similar in their selectivity against Na^+ . As with wild type (Fig. 2a), the mutants showed no detectable inward currents with external Na^+ (not shown). Reversal potentials estimated from the outward currents for wild type and mutants were comparable, ranging from -80 to -90 mV, and indicating a maximum $P_{\text{Na}}/P_{\text{K}}$ of 0.04.

The blocker tetraethylammonium (TEA) has been a useful probe of the vestibule regions of K^+ channels^{3,5}. Currents, measured with two-electrode voltage clamp, were blocked reversibly by extracellular TEA ($K_i \sim 60$ mM in 140 mM Na^+

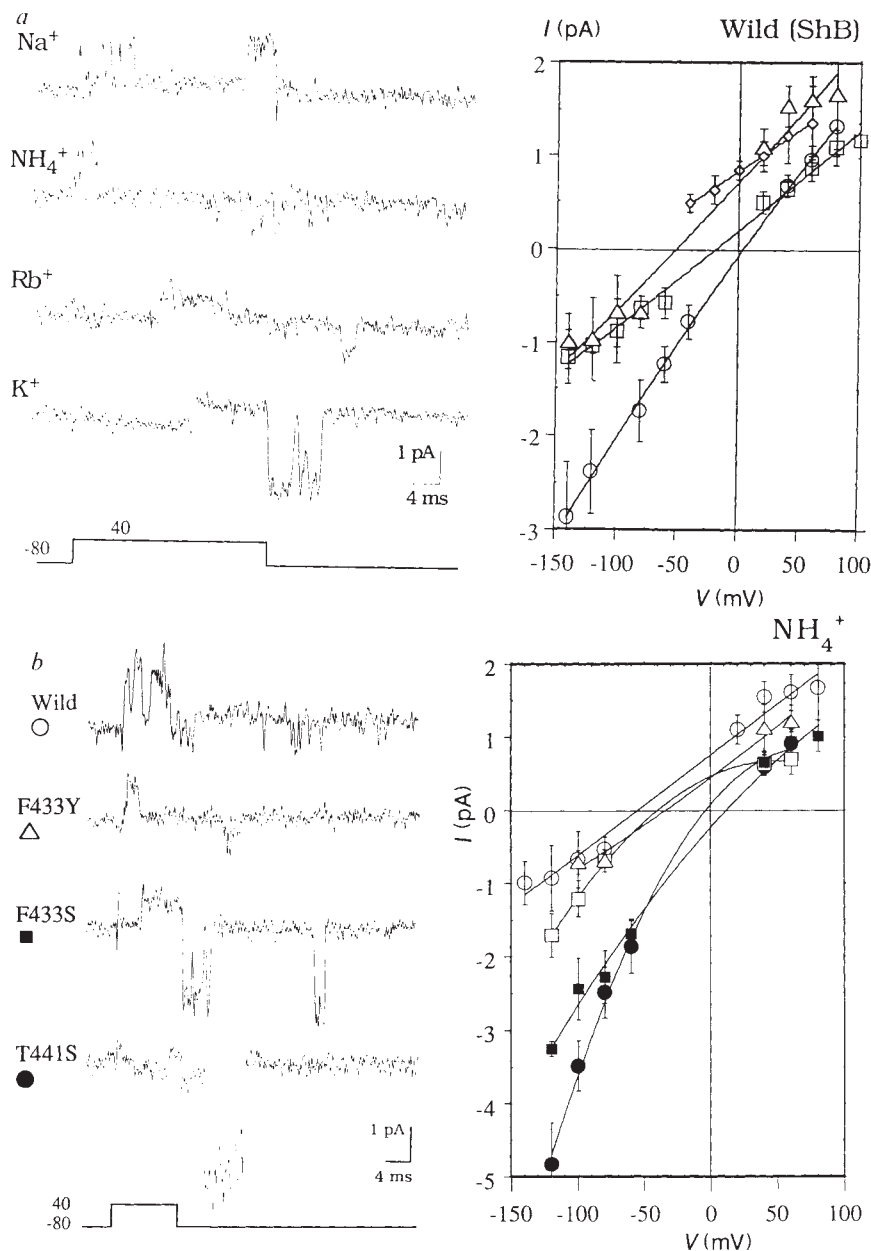


FIG. 2 Ionic selectivity of wild-type and mutant channels recorded in bi-ionic conditions. *a*, Single channel activity of wild-type ShB with K^+ internal and the test ion external (left). The current-voltage relationship (right) shows mean current amplitude $\pm$ s.d. as a function of potential, and illustrates the differences in inward conductance and ionic selectivity for K^+ ($\circ$), Na^+ ($\diamond$), Rb^+ ($\square$) and NH_4^+ ($\triangle$) in the wild-type channel. *b-d*, Single channel activities (left panels) and current-voltage relationships (right panels) for wild-type and mutant channels compared in each bi-ionic condition. The external test ions were: *b*, NH_4^+ ; *c*, Rb^+ ; *d*, K^+ . Current-voltage relations represent data combined from multiple patches for each channel type: wild type ($\circ$), and mutants T441S ($\bullet$), T442S ($\square$), F433Y ($\triangle$) and F433S ($\blacksquare$). Values for *n* are listed in Table 1.

METHODS. Wild-type and mutant cDNA constructs, linearized with *EcoRI*, were transcribed *in vitro* with T3 polymerase. Oocytes prepared from *Xenopus laevis* were injected with 10–25 ng of mRNA in 50 nl of sterile water and incubated 2 or more days at 18 °C before recording¹¹. Single channel activity was recorded at 22–23 °C in cell-attached and inside-out patches with electrodes (2–3 M Ω) filled with Na^+ , K^+ , Rb^+ or NH_4^+ saline. Saline compositions were 100 mM for the Cl^- ion of the test salt, 4.3 mM MgCl_2 , and 3.6 mM HEPES, pH 7.3. In all recordings, the K^+ saline was used as the bath, which depolarized the oocyte membrane potential to -3 to 0 mV in the cell-attached configuration, and provided physiological K^+ for inside-out patches. Channels were activated with tail voltage protocols from holding potentials of -80 or -100 mV. Data were filtered at 2 kHz, sampled at 50 μs , and analysed with pClamp software (Axon Instruments). Mean current $\pm$ s.d. was determined from amplitude histograms. The ranges of mean current amplitudes from cell-attached and inside-out patches were comparable; thus, data from both configurations were combined for analyses. Current-voltage relationships for data combined from multiple patches were fit well by second-order polynomials ($R > 0.98$) which allowed interpolation of the reversal potentials; no mechanistic basis was presumed for the second-order fit.

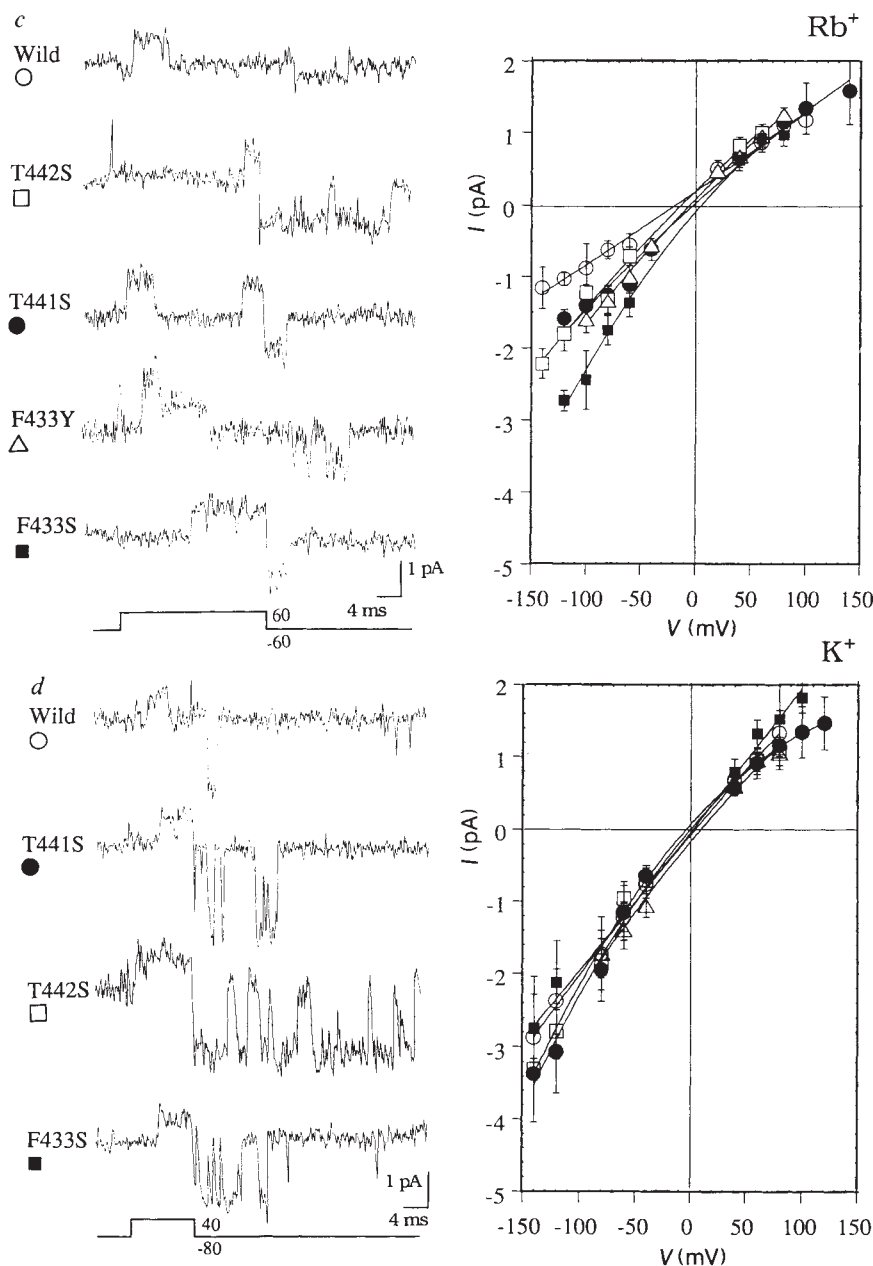
saline; $K_i \sim 30$ mM in 140 mM K^+). None of our mutations detectably altered sensitivity to external TEA (not shown); these amino acids do not seem to contribute to the binding site. In contrast, the previously reported influence of D431 and T449 on external TEA sensitivity suggests that these residues do contribute to the external vestibule⁵.

Most of the mutations characterized here modified channel selectivity, but showed little effect on macroscopic kinetic properties such as rate of inactivation and voltage-dependence of activation and inactivation, measured with two-electrode voltage clamp (Table 1). Values for ShB wild type are comparable to those reported previously^{8-11,17}. The interesting exception is T442S, which had relatively little effect on selectivity, but changed the macroscopic inactivation rate, with the fastest time constant about 100-fold slower than wild type. Voltage mid-points of activation and inactivation were more negative than wild type (Table 1). Single-channel records (Fig. 2) showed prolonged activity of T442S during depolarizing and subsequent hyperpolarizing steps. These effects may reflect stabilization of the open state or decreased inactivation in this mutant.

The changes in selectivity induced by single amino-acid substitutions support the hypothesis that H5 forms the pore-lining

region of the *Shaker* channel. It is unlikely that the selectivity effects result from a general disruption of channel structure as the kinetic, pharmacological and voltage-dependent properties that we measured showed little or no change in the mutants with the greatest influence on permeability. Features of H5 implicate a central role in channel function. This sequence is present in all known K^+ channels of the family characterized by having at least six putative membrane-spanning regions. Many mutations in H5 produced no detectable currents, corroborating evidence from the high sequence homology that this region is intolerant of structural changes. In contrast, large deletions and charge alterations can be made in other regions of the channel^{5,17} without destroying function.

We favour a model in which H5 is tucked into the plane of the membrane and lines the pore. This model is supported by evidence that two amino acids, D431 and T449, strongly influence external TEA binding⁵, and by our data showing that mutations in the intervening region alter selectivity but not external TEA binding. The sites influencing external TEA binding are necessarily extracellular, and define the maximum length of H5. One model that can fit these observations envisions the



sequence of 17 amino acids within H5 (432–448) as a β hairpin that extends ~ 20 Å into the membrane. The juxtaposition of β -hairpins from each subunit of the channel could form the central pore as a β barrel that spans the distance between the internal and external vestibules. β -barrel channel structures have been proposed for some less selective ion channels such as the voltage-dependent anion channel and porins^{18,19}.

Our mutations increased permeability to larger cations, Rb^+ and NH_4^+ , without removing the ability of the channel to exclude Na^+ . These data provide evidence that different structural mechanisms are involved in selecting against cations that are larger or smaller than K^+ . These results are consistent with previous models in which multiple barriers exploit both steric hindrance and differences in ionic charge density^{2,20}. If the

channel complex is a tetramer, changing a single residue would produce a fourfold symmetrical change in the channel. Mutation of threonine to serine, for example, could increase the local pore diameter by the equivalent of two methyl groups. Thus, the flux of larger ions through the channel could be increased if the altered residue contributes to a limiting constriction. The absence of changes in K^+ conductance in our mutants suggests that a binding site of intermediate field strength, not affected by the mutations, remains limiting for K^+ flux but has less influence on Rb^+ and NH_4^+ , which have lower charge densities. Further mutagenesis in H5 and in the equivalent S5–S6 linker of Na^+ and Ca^{2+} channels may reveal the molecular bases of the energy barriers that limit permeation and determine ionic selectivity. □

Received 3 December 1990; accepted 2 January 1991.

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ACKNOWLEDGEMENTS. We thank R. Aldrich, T. Hoshi, E. Isacoff, L. Jan, R. Tsien and W. Zagotta for discussions and I. Inman for technical assistance. This work was supported by the NIH and a Sloan Fellowship to T.L.S. The initial phase of this work was supported by funding from the Howard Hughes Medical Institute to L. Jan, University of California, San Francisco.

Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease

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A LOCUS segregating with familial Alzheimer's disease (AD) has been mapped to chromosome 21 (ref. 1), close to the amyloid precursor protein (APP) gene^{2–5}. Recombinants between the APP gene and the AD locus have been reported^{6–8} which seemed to exclude it as the site of the mutation causing familial AD. But recent genetic analysis of a large number of AD families has demonstrated that the disease is heterogeneous⁹. Families with late-onset AD do not show linkage to chromosome 21 markers^{9,10}. Some families with early-onset AD show linkage to chromosome 21 markers, but some do not^{8,9,11}. This has led to the suggestion that there is non-allelic genetic heterogeneity even within early

onset familial AD^{8,9}. To avoid the problems that heterogeneity poses for genetic analysis, we have examined the cosegregation of AD and markers along the long arm of chromosome 21 in a single family with AD confirmed by autopsy. Here we demonstrate that in this kindred, which shows linkage to chromosome 21 markers, there is a point mutation in the APP gene. This mutation causes an amino-acid substitution (Val → Ile) close to the carboxy terminus of the β -amyloid peptide. Screening other cases of familial AD revealed a second unrelated family in which this variant occurs. This suggests that some cases of AD could be caused by mutations in the APP gene.

Segregation of polymorphic DNA markers spanning 55% of the physical and genetic length of the long arm of chromosome 21 was analysed in family F23 in which early-onset AD has been confirmed after autopsy (Fig. 1). These markers, represented by the loci (from centromere to telomere) D21S16, D21S13, D21S1, APP, D21S17, D21S156 and D21S167 (refs 12–16), cover the genetic distance in which the AD locus has been proposed

TABLE 1 Two-point linkage analyses between Alzheimer's disease and polymorphic DNA markers on the long arm of chromosome 21

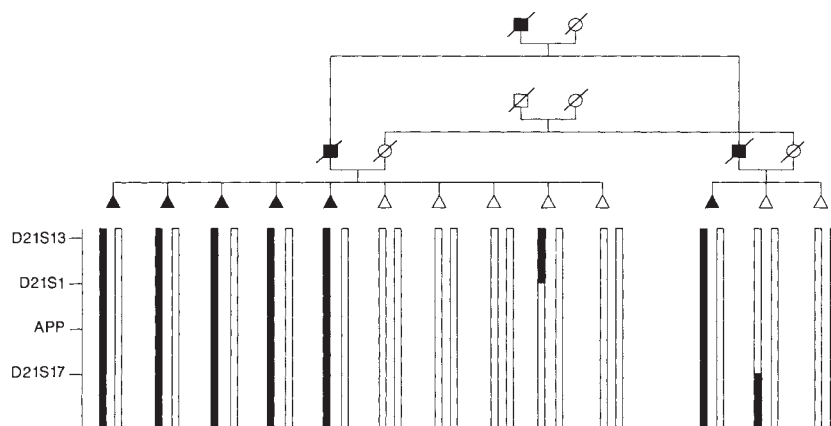
Locus	Recombination fraction (θ)				
	0.00	0.01	0.05	0.10	0.20
D21S16	1.36	1.35	1.28	1.18	0.92
D21S13	2.21	2.17	2.06	1.89	1.44
D21S1	2.65	2.61	2.45	2.22	1.67
APP(EcoRI)	2.93	2.88	2.82	2.36	1.72
D21S17	0.45	0.51	0.61	0.63	0.54
D21S156	−6.31	−4.29	−2.73	−1.76	−0.77
D21S167	−8.02	−3.44	−1.88	−1.17	−0.49
APP(BclI)	3.37	3.31	3.07	2.76	2.09

Allele frequencies used for the loci D21S16, D21S13, and D21S1 have been calculated from unrelated individuals in a British population (M.M. *et al.*, manuscript in preparation). Allele frequencies used for the other polymorphisms are as previously reported^{12–16}. The allele frequency used for the BclI polymorphism was 0.01, which was the highest frequency predicted from non-detection of the polymorphism in 200 normal chromosomes (99% confidence, $[1 - (1 - \text{confidence interval})^{1/200}]$). The following loci were not informative within the pedigree: D21S80, D21S52, D21S11, D21S8, D21S111 and D21S82.

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FIG. 1 Pedigree in which early-onset AD is apparently inherited as an autosomal dominant disorder. The average age of onset in this family is 57 ± 5 yr. Black symbols denote affected individuals and oblique lines indicate individuals who are deceased. Females are denoted by circles and males by squares. Triangles are used in the present generation to preserve anonymity. In generation II the spouses of the two affected brothers were sisters. Samples were available from the 13 individuals whose haplotypes are illustrated, from a further 19 children and spouses of these individuals and from 7 more distantly related unaffected individuals. Beneath the pedigree are ideograms of the two chromosomes 21 in each individual of the third generation at four loci on the long arm of the chromosome. The linkage data suggest that the chromosomes filled in black were inherited from the affected fathers.



to occur, with the exception of the pericentromere. Two-point linkage analyses (MLINK; ref. 17) were carried out between each of the polymorphic loci and AD (Table 1).

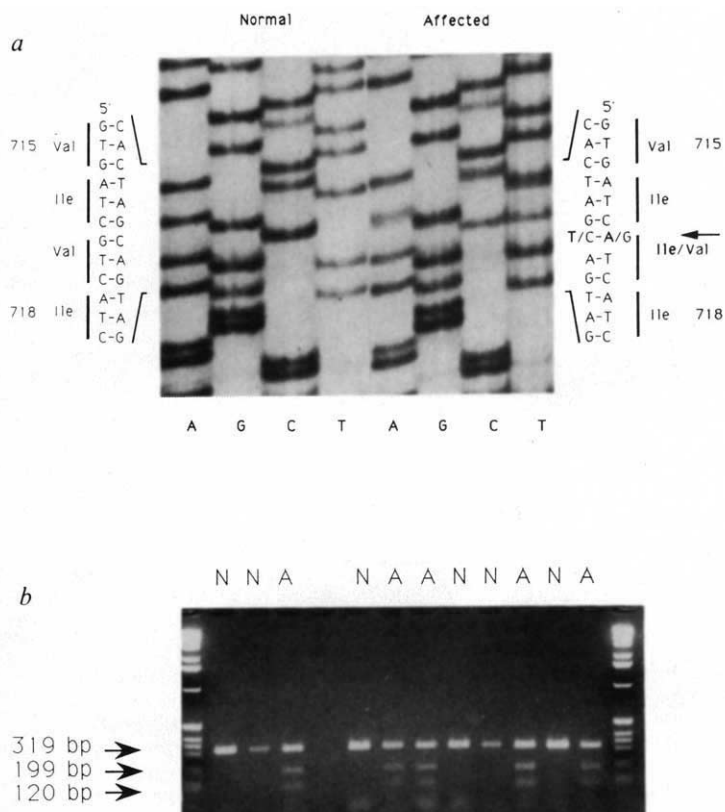
Two individuals with recombinant chromosomes provide evidence for the position of the AD locus (Fig. 1). The unaffected individual in the second sibship who shares the same allele at D21S17 as the affected individuals is 15 years over the mean age of onset of the disease in this family. This recombinant excludes the disease locus from being telomeric to this marker. The individual with a recombinant chromosome in the first sibship is unaffected but is only two years above the mean age of onset within the family (giving odds of two to one against developing the disease based upon the family's risk curve). This person shares the same alleles as the affected individuals between D21S13 and D21S1/S11, but not at APP. If this person were to develop AD in future, then the disease locus must be centromeric of APP and the APP gene is excluded as the disease locus; but

if the person remains healthy, then the AD locus must be telomeric of D21S1/S11 and APP is not excluded.

Because we could not exclude the APP gene as the site of the mutation leading to AD in this family, we analysed the APP gene in an affected family member by PCR direct sequencing using intronic primers^{18,19}. Exon 17 was sequenced first because it encodes part of the β -amyloid peptide and is the site of the mutation leading to hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D)^{20,21}.

Sequencing of exon 17 revealed a C to T transition at base pair 2,149, causing a valine to isoleucine change at amino acid 717 (transcript APP₇₇₀; ref. 19) (Fig. 2a). This valine residue is conserved in rodents. The base substitution may have involved deamination of a methylated cytosine situated 5' to guanine, a fairly common mutation in human DNA²². The substitution creates a *Bcl*I restriction site which allows detection of the corresponding polymorphism within the PCR product (Fig. 2b).

FIG. 2 a, Autoradiograph of a sequencing gel from part of exon 17 of the APP gene in a normal and an affected individual showing a single base-pair change in the affected individual. This C to T transition leads to an amino-acid substitution of a valine by an isoleucine at codon 717. PCR was carried out using the following intronic primers in order to amplify exon 17 of the APP gene: (1) 5'-CCTCATCCAAATGCCCCGTCATT-3', and (2) 5'-GCCTAATTCTCTCATAGTCTTAATCCAC-3'. PCR conditions were 94 °C for 10 min to denature, then 35 cycles of 60 °C for 1 min, 72 °C for 3 min, 94 °C for 1.5 min; and a single cycle of 72 °C for 10 min. The reaction volume of 25 μ l contained 50 pmol of primers, dNTPs at 200 μ M, and $MgCl_2$ at 1.5 mM. A second PCR was performed which contained 50 pmol primer (1) and 0.5 pmol primer (2). The PCR product was purified on a Centricon 100 microconcentrator (Amicon) and used directly for sequencing with the Sequenase kit (version 2.0) (USB) following the manufacturer's protocol. Direct sequencing of exon 15 has revealed that this exon sequence is normal in family F23. Single-strand conformational analysis has failed to detect any sequence alterations in exons 7, 15 or 16 (our unpublished data). b, *Bcl*I digests of the exon 17 PCR product from unaffected (N) and affected (A) individuals in an early-onset AD family showing cosegregation of the restriction site and the disease. *Bcl*I digests were carried out at 50 °C for 2–4 h then electrophoresed in 3% agarose and stained with ethidium bromide. Size markers (indicated in base pairs) are shown in the outside tracks of the gel.



Embryonic lethality caused by mutations in basement membrane collagen of *C. elegans*

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BASEMENT membranes are specialized forms of extracellular matrix with important functions in development¹⁻³. A major structural component of basement membranes is type IV collagen, a heterotrimer of two $\alpha 1(IV)$ and one $\alpha 2(IV)$ chains, which forms a complex, polygonal network associated with other basement membrane components^{4,5}. Here we report that the $\alpha 1(IV)$ collagen chain of *Caenorhabditis elegans* is encoded by the genetic locus *emb-9*. Mutations in *emb-9* cause temperature-sensitive lethality during late embryogenesis. We have identified single nucleotide alterations that substitute glutamic acid for glycine in the triple-helical Gly-X-Y repeat region of the $\alpha 1(IV)$ collagen in three *emb-9* mutant strains. These results are direct evidence that defects in basement membranes can disrupt embryonic development and form a basis for the genetic analysis of basement membrane function.

The basement membrane collagen genes *clb-1* and *clb-2* from *C. elegans* are homologues of the human $\alpha 2(IV)$ and $\alpha 1(IV)$ genes respectively⁶. The gene *clb-2* was localized on the physical map of linkage group III, roughly 180 kilobases (0.2 genetic map units⁷) to the right of *lin-12*. Assuming that mutations in basement membrane collagen would be lethal in embryos, we examined seven embryonic lethal genes (*emb-9*, *emb-24*, *emb-25*, *emb-30*, *emb-33*, *emb-34* and *zyg-7*) located near *clb-2*. These genes were identified in random screens for temperature-sensitive embryonic lethal mutations⁸⁻¹¹. One gene, *emb-9*, was highly mutable (five random alleles) and all five alleles were semi-dominant. These properties have been noted for other collagen genes: the human type-I collagen genes are apparently highly mutable^{12,13}, and both human^{12,13} and *C. elegans*^{14,15} collagen genes can have frequent dominant alleles. At restrictive temperatures, *emb-9* embryos arrest during the morphogenetic phase of embryogenesis, displaying gross morphological defects. Larval animals shifted to restrictive temperature arrest development or become subfertile adults, indicating that *emb-9* function is necessary throughout development.

FIG. 1 Structure of the *C. elegans* $\alpha 1(IV)$ collagen gene *clb-2*. Exons are indicated by boxes, introns by horizontal lines. The Gly-X-Y repeating triple-helical domain is lightly shaded, coding sequence preceding the helical domain is heavily shaded, and the NC1 domain is cross-hatched. Interruptions of the Gly-X-Y repeats are depicted as solid vertical bars. The locations of the nucleotide alterations in the three identified *emb-9* alleles are shown at the bottom of the structure. A nucleotide scale is shown in kilobases, numbering the A of the initiator ATG as +1. Restriction site symbols are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sac*I. The *Sac*I site inactivated by the *g34* allele is shown in bold face. The *Bam*HI sites that define the probe used for RNase protection (see Fig. 2) are in bold face. The precise locations of the beginning of the first exon and the end of the last exon have not been determined. The complete nucleotide sequence of *clb-2* is available on request EMBL Accession No. X56979.

METHODS. The nucleotide sequence was determined from plasmid subclones of the genomic phage clone CH 2 (ref. 6). Exonuclease III deletion clones²²

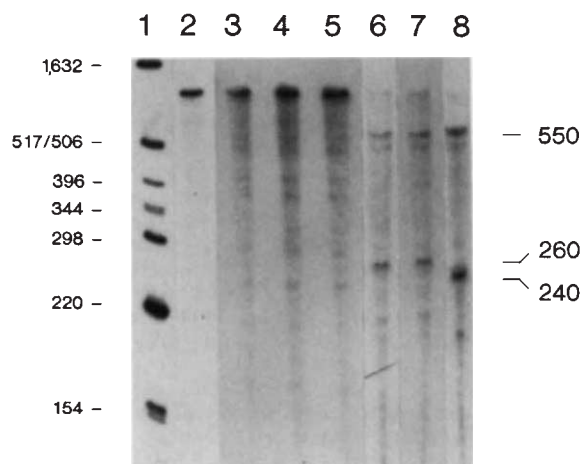
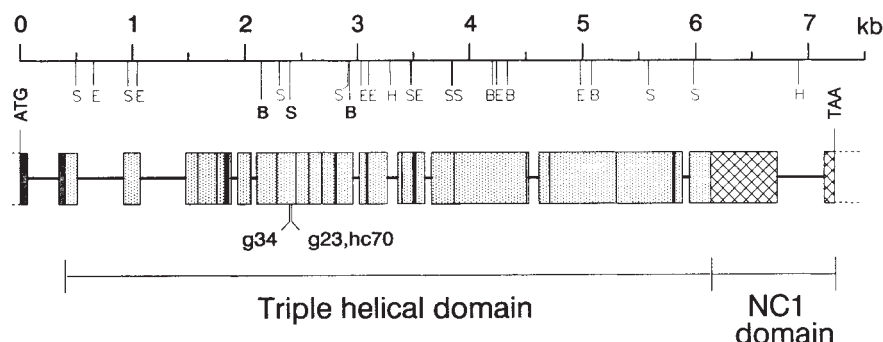


FIG. 2 Identification of nucleotide alterations within *clb-2* in genomic DNA from *emb-9* mutant strains. Lane 1, molecular weight standards; numbers of base pairs indicated to the left. Lane 2, untreated complementary RNA probe. Lanes 3-8, protected fragments obtained using genomic DNAs from the wild-type N2 strain (lane 3) and from animals carrying the *emb-9* mutant alleles *b117*, *b189*, *g23*, *hc70* and *g34* in lanes 4-8, respectively. The *g23* and *hc70* nucleotide alterations appear to be located at the same site, generating cleavage products of 550 and 260 nucleotides. The *g34* alteration is located ~20 nucleotides away. Sizes are indicated in base pairs.

METHODS. Radiolabelled cRNA probe was produced from a clone of the 0.8-kb *Bam*HI fragment (nucleotides 2,149-2,932) in the Bluescribe plasmid vector (Stratagene). The cRNA probe was annealed to 5 μ g of denatured genomic DNA from the various strains and the resulting RNA-DNA hybrids were treated with RNase A as described¹⁶. Resistant products were analysed by electrophoresis on a 4% acylamide/urea gel, which was dried and exposed to X-ray film. No other nucleotide alterations were detected in any of the five *emb-9* mutant DNAs using cRNA probes covering the region from the *Sac*I site (Fig. 1, nucleotide 954) to the *Hind*III site (nucleotide 6,916) near the end of the gene.

To determine whether *emb-9* was the $\alpha 1(IV)$ collagen gene *clb-2*, we tested the five *emb-9* mutant strains for nucleotide alterations in *clb-2*. A *Sac*I restriction site was missing in DNA from animals carrying the *g34* allele (Fig. 1). Alterations were detected in the *g23* and *hc70* alleles using RNase protection¹⁶ (Fig. 2). Identification of alterations within *clb-2* in three *emb-9* mutant strains strongly suggested that *emb-9* was *clb-2*.

In support of our assignment of *clb-2* as the homologue of the human $\alpha 1(IV)$ collagen gene, an alignment of their amino-



and specific oligonucleotide primers were used to complete the sequence on both strands. The locations of the nucleotide alterations in the *g34*, *g23* and *hc70* alleles of *emb-9* were determined from restriction mapping data, RNase protection experiments (Fig. 2) and sequencing of mutant DNAs (Fig. 4).

FIG. 3 Alignment of *clb-2* and the human $\alpha 1(IV)$ collagen²³⁻²⁵ amino-acid sequences, excluding NC1 domains. Identical amino acids are indicated by double dots, similar amino acids by single dots. Aligned cysteine residues are marked with shaded bars. The beginnings of the Gly-X-Y repeats are indicated with arrows (amino acid 43 in both sequences). Interruptions of the Gly-X-Y repeats are underlined. The locations of the glycine (G) to glutamic acid (E) alterations identified in the three *emb-9* mutant alleles are shown above the sequence of *clb-2* (amino acids 402 and 408). Alignment of the NC1 domains was presented previously⁶. The percentages of amino-acid sequence identity between *clb-2* and the human $\alpha 1(IV)/\alpha 2(IV)$ collagens are: triple-helical plus amino-terminal domain 44/40, NC1 domain 62/58, complete sequence 47/43.

METHODS. Alignment of the amino-acid sequences used the PALIGN program (Intelligenetics) using the structure-genetic matrix comparison, open gap cost = 10, unit gap cost = 5. The amino acids indicated as similar are: A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W (single-letter code).

acid sequences is presented in Fig. 3. Notably, the five cysteine residues in the amino-terminal 7S domain, involved in formation of tetrameric type-IV collagen complexes^{4,17}, are conserved. There are 17 interruptions of the Gly-X-Y repeats in *clb-2*, ten at the same positions and three close to the positions of interruptions in the human collagen. In all comparisons, *clb-2* has greater similarity to human $\alpha 1(IV)$ than $\alpha 2(IV)$ collagen. These similarities of structure and sequence support the assignment of *clb-2* as the homologue of the human $\alpha 1(IV)$ collagen.

The three *emb-9* alterations were localized to the triple-helical domain of *clb-2* (Fig. 1) and their precise sequence changes were determined (Fig. 4). All three of the mutations were G → A nucleotide changes, which would result in replacement of the glycine in a Gly-X-Y repeat with glutamic acid. These results are evidence that *emb-9* is the genetic locus representing the $\alpha 1(IV)$ collagen *clb-2*. Further confirmation was obtained from the ability of the wild-type *clb-2* clone to rescue the lethal phenotype of *emb-9* in transgenic animals (Fig. 4 legend). The *g23* and *hc70* mutations are identical and located 18 nucleotides from the *g34* mutation. The close proximity of these mutations indicates that there may be limited regions of the $\alpha 1(IV)$ collagen gene that can be mutated to generate the temperature-sensitive embryonic lethal phenotype these alleles were selected to display. Although we have not localized the two remaining *emb-9* mutations, they are apparently in a different region, indicating that at least one other region can yield such a phenotype. Alterations in other regions of the gene may most commonly result in non-conditional lethality, non-lethal phenotypes, or display no phenotype.

These mutations create interruptions of the Gly-X-Y amino acid repeats, replacing glycine with glutamic acid. Although type IV collagens normally contain many interruptions of their

<i>clb-2</i>	MS-RLSLLGLTAAYVLLSSFCQDRIHVDAAAKGKCAPPCVPGTIGKGRNPGFGGEPGHPAGPGDQGPAGP	73
Human $\alpha 1(IV)$	MGRLSVMLLLPAAALLHEHNSRAAAKGGCAGSGCC-CDCGHVKGOKGERGLPGQQVIFPGMGPEPGP	73
	APGMFAEGDQDMGSKGARGDLPGSPGHPGLDGLDGLPGKEEGIPGCGNDGFGPMPLAGPPGQSGON	147
	PPGOKDGTGEPGLPGTKTRGPPGASGYPGNLPLGIPGDDGPPGPPGICGNGTKGERGLDPPGLPGFAG--	145
	GMPGRPLSGPPGEGGVNSGGRKGVKGSRGVPLPGMSYPLGKAKDGPYGLPGFPVSGLGKRMGVR	221
	-NPPGPPGLPMKGDGPE-----LGHVPMMLKGERGFGIPGTPGPPGLPGLQGP--VG	197
	TSVKGKGLPGPPPPGQSPYHASKPIEMELQGVPGVAGVKEGGRDGPVPPGMLGLOGPPYPGLKGO	295
	PPGFTGPPGPPGPPGEGQMLSFQ-----GPKGDKQGVSGPPVPGQAQVQEKGFAT-KGE	259
	KDGLDGLDKGRKDGVPVNGYKESQGEGLGGTPGPGTKGAGEPGYPGRPFEGDQCGEPGLGEGTEAG	369
	KGQKGEFG-----GMPGVEGKEGPGKGRKPKGDGKGEKSGPFGPEGPGLIGRQGPQGE-KGEAG	326
	(g34)E E(g23, hc70)	
	PHGAQGDGVQGGKGLPGHDLPGVPGRPGVPGAPGAPGIDGMPGYTEKGRDGEDGYPGAFGLPGEPG	443
	PPGPPGIVTIGLGEKGERGYPGTPGRGEPGKGFPLPGAPGPPPLV-----PGQAGAPGPPGERG	392
	DCGYTGEDGLGYDGGPPGLDQSGRGGFPGIPDGDGPGYSGEKGFGPGVKNYKPPGMPGLPGEPGMPRI	517
	E--KGRGFPGLSGPSGRDGLPGPPGSGPPGQ-----PGYTNIGVECPGPPGQGGPPGIP	448
	GVDGYPGPPGNGGERGDCGYCPDGVPGNAGDPGPGMNGYPPGPPGNDHGDGMPGAPGKPSAGSDGLSGS	591
	GQGFIGEIGEGKQKESCLIC-----DIDYRGPPGQPPGEIGFGAPGAKGRGLPGRDGVAGVPGP	514
	PLGLPIPGYPMKGEAGEIVCPMENPAGIPGLKDHGLPLGPRPSDGLPGYPPGPGNGFPLGQEPGLAGI	665
	QGTPLIGOPGAKGEPGEFFD-----LRLKDKGDPGPGPGMPGRAGSPGRDGHPLPGPKSPGVSGL	581
	DGKRGRQSLGIPGLGPPDQSPFGPPTGPGYKGERADGLPGLPGAQPRGIPAPLRIVNOVAGQPPGVDMPG	739
	KGERGPPGGVGFPSRGDTGPPGPPGYGAPGIDGKQAGFPGGGSPGLPGKPEPKIVPLPGPPGAELGL	655
	LPDGRADGLPLGPPVPGDGYPTGEPGMDGLPGFPGLHGEPMGRQGGVFNIGDQCGEPGLDGYGPAP	813
	SPGFPGQGRGFPPTGPRGLPGEKAVGQPGI---GFPGPPGKVDGLPGDMGPPPTGPRGPNGLGPNP	725
	GAPGAPGETGFGFGDQGYGPNAGAAAGLPGDPGYPGRDLPGTPGYGAEAGNMGQAGQPPGSRGSEGLV	887
	GVQKQKGEPEGLGLKGLPLGIPGTPGEGKSTGVPGVPEHGAIGPPGLQIGRGEPPGLPGSVGSPGPV	799
	GIDGKGRDGTGTRGDDGPGYSGEAGAPGNGMDGYPG--APDQGYGSPGDDGYPPGSGIPGEDGLVGF	959
	GI---GPPGARGPPGGQPPGLSGPPGIGKEKGFPGFPLDMPGPKGDKGAQGLPGITGOSGLPLPGQAGP	869
	GLRGEHNDGLPGLGECGEGSRGLDGVPGYPGHEHTDGLPLPGADGQPGFVGEAGEPTGYPGRQPPGPN	1033
	GIPGFGSKGEMGMTPGAPGSPGVPAGPLGPKGKHGFPKSGPRDGLKDKQDVLGPKGSMMDKVDN	943
	LAYPGQDQVYPPGPPGLPGDGLPLNGERDNDST--PGMPLSGQDAGYDGLDGVPPGPPYPTIG	1106
	GSMLGKQDQGEKQIGIPGEGSRGDPGTPGVPGDQGAQPGGPGKDGPGISGTPGAPGLPGPPGVSQMG	1017
	MPGLKGESGLPLGPRQDNDGIPGQPLGECGEDGFPSPGPGYPPGQGRGEGKYPGIPGNGPLGLRQD	1180
	LPPTGEGKVPPIPGPQSGPLPGDKAKGEGQAGPPGII-GIPGLRGEKQDQIGFPGSPGKEKSGISIP	1090
	GQPLKGENLDGQPGYPSAGQLGTPGVYGPAGGEMDNGRQDGPGLRGSQPGQPPGLPRDQGP	1254
	GMPGSPGLKSPGSGVYGPGLGPKGDKGLPLDGLIPGVKGEAGLPGTPPTGAGQKGEPSDGIPIGSAGE	1164
	VGPPGDDGYGAPGQDLYPPGGAAGDGYPLDGLPAGPLNGEPSPGQYMPGLPGGPGESGLPGYPGERGL	1328
	KGEPLGRGFPFGPAKDKGSKGEVGFPLAGSPGIPGSKGEGFMGPPGQGGPLPGSPGHAT-EGPKD	1237
	PLDGRKHGDLPGAPGVGVEGVPGLGECGEDGYPGAPAGPSNGYPPGERLPGVPQGGRSQDNGYGPAGP	1403
	RCPGQAPGLPLGPPGPPPLGIDGVKDKXMPGWPAGVPGPKDQPGQMPGIGGSPGIGSKGDMGPPG	1312
	QPGIKGPRGDDGFPGRDGLDGLPGRGREGPLGPNAMAVRMPGQGGYPPGKGYPLPGDMGLSGPPKAG	1476
	VPGFQKPLGLQGLKGDQGGQVPGAKLPGPPPPGYPDIK-GEPLPGPEGPPGLKQGLPGPKQGG	1384
	YPGAPGTGYPGPPGLSGMPGHGDDGFGQAGRTGNPLGPT---PGYPSGPGW	1529
	VTGLVGIPGPPGIPGFGAPGKQKEMGAPGTPGPRGPPGPPGDLPGSMGPPGTP	1440
		NC1 DOMAIN

FIG. 4 Partial nucleotide sequences of the wild-type *C. elegans* $\alpha 1(IV)$ collagen gene and *emb-9* mutant alleles. Nucleotide changes in the mutant alleles are shown above the wild-type sequence, the deduced amino-acid changes are shown below the sequence. Because it is unusual for two randomly selected mutations to be the identical nucleotide change, the identity of *g23* and *hc70* was reconfirmed. The *SacI* restriction site that is inactivated by the *g34* mutation is underlined. Nucleotide numbering refers to Fig. 1 and numbering of amino acids refers to Fig. 3.

METHODS. Mutant sequences were determined from plasmid clones of the 0.8 kb *Bam*HI fragment (nucleotides 2,149-2,932) derived by polymerase chain reaction amplification²⁶ of genomic DNAs from the *emb-9* mutant strains. Multiple clones derived from each strain were sequenced and only the single nucleotide alterations shown were found. Transgenic rescue: Transgenic animals were generated using a modification of previous pro-

	A (g34)	A (g23, hc70)	
2377	GGTCCAAGAGGTCCAGTTCGAGCTCAGGAGCCCCAGGACAGCCCGTATCGATGGAATGCCA		2440
396	GlyProArgGlyProValGlyAlaProGlyAlaProGlyGlnProGlyIleAspGlyMetPro		416
	Glu	Glu	

cedures (ref. 27; C. Mello, J.M.K., D. Stinchcomb and V. Ambros, manuscript in preparation) A mixture of CH 2 and pRF4 DNAs was microinjected into the gonads of *g23* or *g34* animals. CH 2 is the genomic phage clone containing the wild-type *clb-2* gene⁶ and pRF4 is a plasmid carrying the dominant right roller cuticle collagen allele *rol-6(su1006)*¹⁵ which is used as a visible marker for transformation. Injected animals were placed at 20°C, a temperature that results in larval arrest of *emb-9* animals, and RRo adult offspring were picked. Several transgenic lines were generated from both *g23* and *g34* animals; other alleles were not tested. At 25°C these lines are viable and fertile, but segregate some lethal and sterile offspring, presumably as a result of loss of extrachromosomal transgenic arrays.

the human, *C. elegans* and *Drosophila*¹⁸ $\alpha 1(\text{IV})$ collagens. The interruptions created by the *emb-9* mutations probably interfere with stable triple-helical assembly of type-IV collagen chains, as in human type-I collagen¹⁹.

A novel $\alpha 5(\text{IV})$ collagen chain has been identified in humans²⁰ and is the basis of Alport syndrome²¹, which causes progressive postnatal glomerulonephritis and hearing loss. These localized effects may, at least in part, reflect localized expression of the $\alpha 5(\text{IV})$ collagen²⁰. The defects in embryogenesis and larval growth that result from mutations in the *C. elegans* $\alpha 1(\text{IV})$ collagen gene indicate that it is needed throughout development and that its expression may be more general. Similar mutations in the broadly expressed human $\alpha 1(\text{IV})$ collagen gene are also likely to result in embryonic lethality. □

Received 12 October; accepted 10 December 1990.

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ACKNOWLEDGEMENTS. We thank C. Mello for his assistance in generating transgenic lines and for lines that he created. Some of the strains used in these studies were provided by the *Caenorhabditis* Genetics Center, which is supported by the NIH Division of Research Resources. This work was supported by the NIH.

Novel myosin heavy chain encoded by murine *dilute* coat colour locus

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HUNDREDS of murine *dilute* mutations have been identified and analysed, making *dilute* one of the best genetically characterized of all mammalian loci. The recessive *dilute* (*d*) coat colour mutation carried by many inbred strains of mice produces a lightening of coat colour, caused by an abnormal adendritic melanocyte morphology that results in an uneven release of pigment granules into the developing hair shaft^{1,2}. Most *dilute* alleles (*dilute-lethal*) also produce a neurological defect, characterized by convulsions and opisthotonus, apparent at 8–10 days of age and continuing

until the death of the animal at 2–3 weeks of age³. The discovery that the original *dilute* allele (now termed *dilute-viral* or *d^v*) is the result of the integration of an ecotropic murine leukaemia provirus⁴ has allowed the cloning of genomic DNA^{5,6} and in this study complementary DNA, from the *dilute* locus. The predicted *dilute* amino-acid sequence indicates that *dilute* encodes a novel type of myosin heavy chain, with a tail, or C-terminal, region that has elements of both type II (α -helical coiled-coil) and type I (non-coiled-coil) myosin heavy chains. *Dilute* transcripts are differentially expressed in both embryonic and adult tissues and are very abundant in neurons of the central nervous system, cephalic ganglia, and spinal ganglia. These results suggest an important role for the *dilute* gene product in the elaboration, maintenance, or function of cellular processes of melanocytes and neurons.

The nucleotide and deduced amino-acid sequences of brain cDNA from the wild-type *dilute* locus are shown in Fig. 1. The nucleotide sequence contains a single long open reading frame capable of encoding a protein of 1,852 amino-acid residues with an unmodified relative molecular mass of 215,000 (*M_r*, 215K). Antibody directed against the C-terminal region of the putative *dilute* protein recognizes a protein of the appropriate size in cells wild type at *dilute* (M.C.S., P.J.D., N.G.C. and N.A.J., manuscript in preparation). In addition, the identification of a discrete intragenic deletion in a *dilute-lethal* allele⁶ provides further evidence that this cDNA represents a transcript from the *dilute* locus. A computer search for amino-acid sequence similarity with other cloned genes indicated that the putative *dilute* protein is a myosin heavy chain (MHC) with a novel C-terminal region.

Myosins bind actin and produce mechanical force through ATP hydrolysis⁷ and are composed of one or two heavy chains (MHCs) and one to four light chains. The N-terminal, or head region, of the MHC changes conformation in the presence of actin and ATP to produce force. Myosin IIs (two-headed) include the conventional myosins that participate in muscle contraction and cytokinesis. Dimerization of the two heavy chains is promoted by the α -helical coiled-coil C-terminal, or tail, region. Myosin Is (one-headed) consist of a single MHC lacking an α -helical coiled-coil tail complexed with one or more light chains. Accumulating evidence suggests that myosin I isoforms are associated with cell membranes where they provide force for movement of membranes along actin filaments^{8,9}. The cellular movements powered by myosin I have been suggested to include pseudopod extension, membrane ruffling, and organelle movements.

The N-terminal half of the predicted *dilute* amino-acid sequence (residues 1–887) shows extensive homology with the head regions of MHCs, including the ATP- and actin-binding sites (Fig. 2a). Five imperfect tandem repeats are present at the *dilute* head–tail junction (Fig. 1, residues 776–887; Fig. 2b). Three similar tandem repeats are present at analogous locations in chicken¹⁰ (not shown) and bovine intestinal brush border myosin type I heavy chain (BBMHCI)¹¹ (Fig. 2b). BBMHCI, complexed with three to four calmodulin light chains instead of the conventional regulatory myosin light chains, tethers the central actin bundle within each microvillus to the plasma membrane^{12,13}. The region of BBMHCI containing the three tandem repeats has been implicated in calmodulin binding^{14,15}. A single copy of this sequence is also present in other MHCs, such as *Dictyostelium* MHCII and rat skeletal muscle MHCII. This sequence has been implicated as a binding site for regulatory myosin light chain¹⁶. The presence of these sequences suggests that the *dilute* protein may associate with multiple calmodulin or myosin light chains.

The C-terminal, or tail, sequence of the predicted *dilute* protein is different from those found in MHC I and MHC II sequences. The five tandem repeats are followed by an α -helical coiled-coil sequence (Fig. 1, residues 914–1,040) which has the characteristic heptad repeat of hydrophobic and basic residues found in other α -helical coiled-coil sequences¹⁷ (Fig. 1). The

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size from those seen in other mouse tissues. Shorter, less abundant transcripts are also observed in most tissues. These transcripts are not detected with a tail region-specific probe (Fig. 3). Although these transcripts may be encoded by other MHC loci related to *dilute*, low stringency southern blot hybridization has so far failed to identify any such loci (data not shown).

The complex transcription pattern of *dilute* could result from differential use of 5' initiation sites, 3' poly(A) addition signals, or alternative splicing. We have not yet obtained any evidence for alternative splicing in brain cDNA, suggesting that the differences between the three major transcript sizes result largely from differential use of 3' poly(A) addition signals or 5' initiation sites. Consistent with this view, two poly(A) addition signals have been identified in the cDNA sequence (Fig. 1). Whether the smaller, less abundant transcripts or the unique transcripts present in kidney result from differential splicing remains to be determined.

In situ hybridization analysis of *dilute* expression in wild-type adult brain is shown in Fig. 4a. Hybridization was localized to neuronal cell bodies throughout the brain. Neuronal expression can be more easily visualized in the higher power magnification shown in Fig. 4c. Hybridization to a myelin basic protein probe, expressed in glial cells within the brain, was included as a control (Fig. 4b). In 14.5-day *post coitum* embryos, the highest levels of *dilute* transcripts were present in spinal cord and in cranial and spinal ganglia, with lower levels observed in skin and brain (Fig. 4d). Hybridization to brain and spinal cord was also observed in 9.5, 11.5, 12.5 and 13.5 days *post coitum* (data not shown).

The lack of melanocyte dendrites associated with all *dilute* mutations suggests that the *dilute* gene product is required for the elaboration, maintenance, or function of cellular processes in melanocytes. The neurological phenotype of most *dilute* mutations and the expression data described in this study suggest

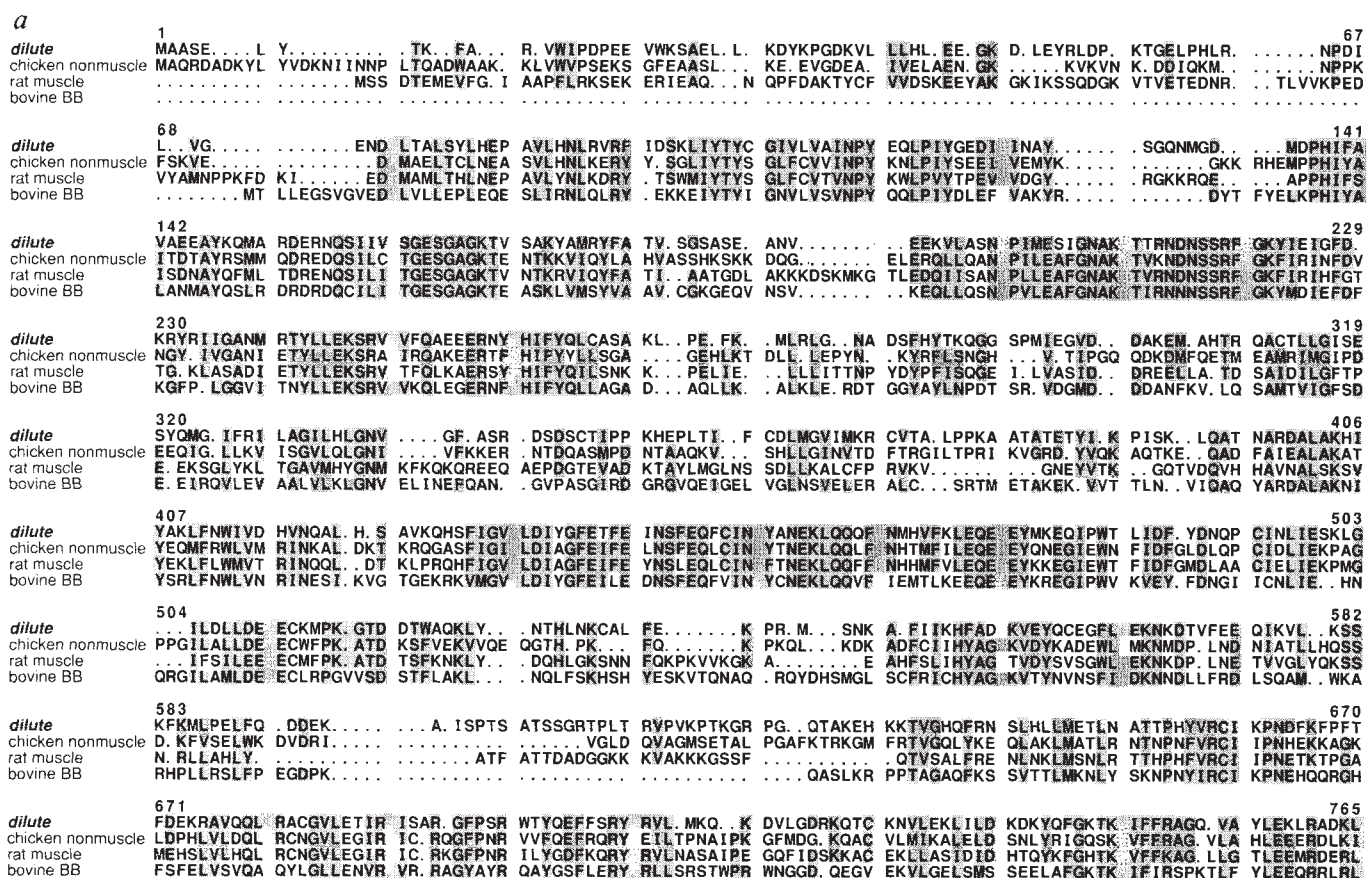


FIG. 2 a, Alignment of the deduced *dilute* amino-acid sequence with the head (S1) sequences of chicken nonmuscle MHCII²⁶, rat skeletal muscle MHCII²⁷, and bovine intestinal brush border MHCII¹¹, using the GAP program²⁸. Dots represent gaps inserted to optimize alignment. Identical or conserved residues among at least three of the four sequences are shaded. Numbering refers to the *dilute* sequence. b, Alignment of repeats from the *dilute* sequence with corresponding regions of bovine BBMHCII¹¹, *Dictyostelium* MHCII²⁹, and rat skeletal muscle MHCII²⁷. Residues that are identical or conserved among at least four of the five *dilute* repeats are shaded. Sequences are aligned with respect to the *dilute* repeats. Groups of conserved residues are defined as A and G; S and T; N and Q; D and E; K and R; L, M and V; F, W and Y. c, Schematic representation of the domain structure of the putative *dilute* protein.

b

RAACIRIGKTIRGHWLLRKRYLCMQ
RAA ITVQRYVRGYQARC YAKFLRR
KAA TTIQKYWRMYVRRRY KIR
RAATIVIQSYLRGLYLRNRYRKILRE
YKA VTIQKRVRGWNLARTHYKRTMKA

dilute
(766-887)

QQLATLIGKTYRGWNRCTHYQLM
RKSIQIVISSWFRGNMOKKHRYK
KASALLIQAFVRGNKARKNYRKY

bovine BBMHCII
(698-766)

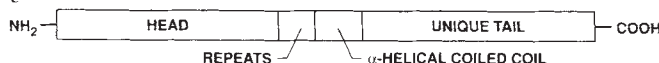
SEIIKAIQAATRGWIRKVVQKAREH
TVAARI IQQLNR

Dictyostelium MHCII
(763-800)

AKLITRTQAVCRGFLMRVEYQKMMQR
RESIFCIQYNIIRAFM

Rat skeletal muscle MHCII
(783-823)

c



that the *dilute* gene product may have a similar role in cellular processes of neurons. Nonconventional myosins have previously been proposed to be responsible for the mediation of retrograde movement of actin filaments or the anterograde movement of membrane antigens during neurite outgrowth^{8,18-20}. The *dilute* gene product therefore represents an attractive candidate for

the mediation of some aspect of axoplasmic transport. Unlike the obvious abnormalities observed in melanocytes of homozygous *dilute-lethal* mice, neither the peripheral nor central nervous systems of such mice display striking histopathological deficiencies. Thus, during development of *dilute-lethal* mice, most neurons appear to elaborate axonal and dendritic processes successfully. Therefore, the behavioural abnormalities and premature death of *dilute-lethal* mice presumably arise from either an abnormality in neuronal function or from failure in the development or maintenance of a critical, as yet undefined, neuronal population.

Overlapping function in cytoskeletal components has been hypothesized²¹ and experimental evidence for the redundancy of myosin I isoforms has been obtained in *Dictyostelium*²². The results described in this study are also consistent with the existence of a family of *dilute*-like MHCs with largely overlapping functions, as expression of wild-type *dilute* product does not seem to be essential for development of most of the nervous system.

Additional studies are in progress to isolate other MHCs with functions related to those of *dilute* and to determine the nature and cellular localization of *dilute* gene products. The hundreds of *dilute* mutations (either spontaneous or induced by X rays and chemicals) ranging in phenotype from slight dilution of coat colour to lethality and the epistatic interactions between the *dilute* suppressor locus and the *ashen*, *leaden*, and *dilute* loci^{23,24} provide extraordinary resources for the genetic dissection of *dilute* function in mammalian development. □

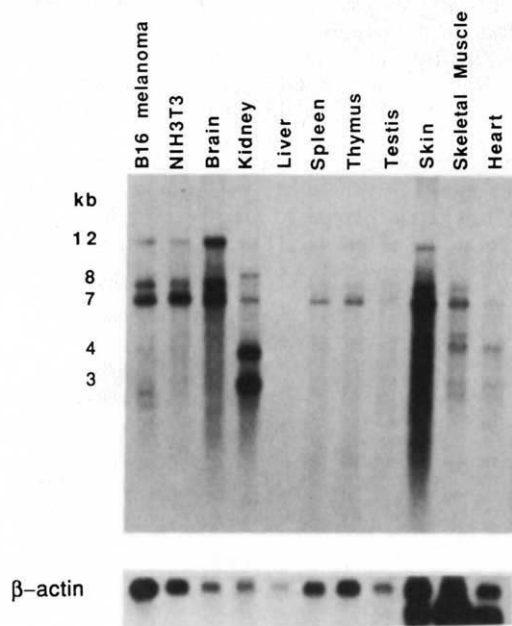


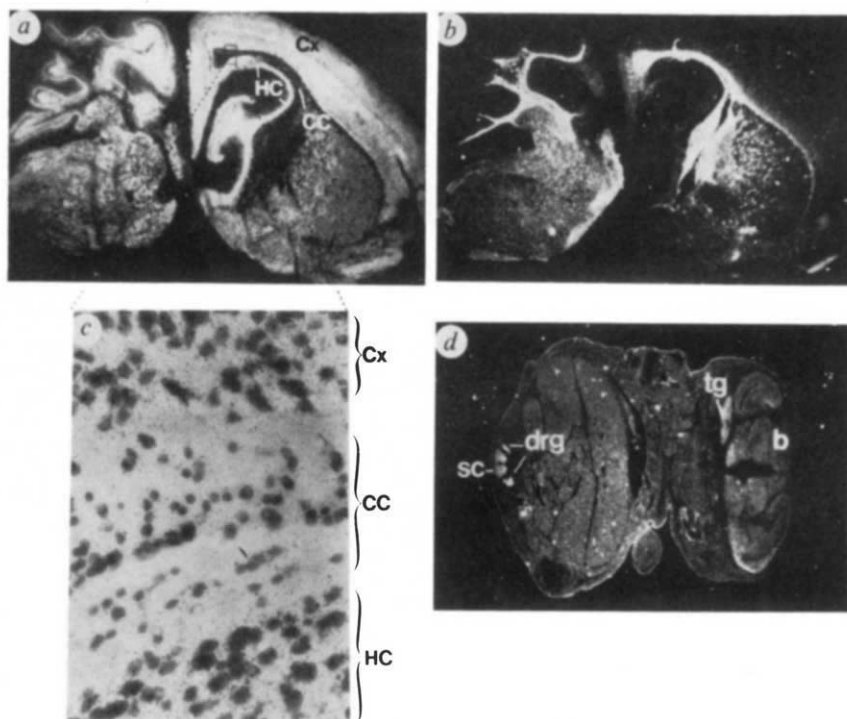
FIG. 3 Northern blot analysis of poly(A)⁺ (2 µg per lane) B16 melanoma, NIH3T3, and 3-week-old C57BL/6J mouse RNA, hybridized with a mixture of *dilute* head (nucleotides 527–992, Fig. 1) and tail (nucleotides 4,111–5,630, Fig. 1) region cDNA probes. The tail region probe alone does not recognize the smaller, less abundant transcripts observed in all tissues (data not shown). A probe from the 3' untranslated region of the cDNA sequence (nucleotides 6,190–6,911, Fig. 1) does not hybridize to the 7-kb transcript, suggesting that the poly(A) addition signal at nucleotide 6,069 is used for the 7-kb transcript (data not shown). The same northern blot hybridized with a control β -actin probe is shown beneath.

Received 9 October; accepted 18 December 1990.

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FIG. 4 *In situ* hybridization analysis of *dilute* expression. *a*, Dark-field photograph of parasagittal section of adult wild-type (C57BL/6J) brain hybridized with a *dilute* cDNA probe (nucleotides 3,837–7,089, Fig. 1). *b*, Dark-field photograph of adjacent section to *a* hybridized with a myelin basic protein cDNA probe³⁰ as a control. Note the nearly converse pattern of hybridization when compared with panel *a*. *c*, Higher magnification bright-field photograph of the region boxed in *a*, illustrating neuron-specific expression of *dilute*. Neuronal cell bodies in the cerebral cortex (Cx) and hippocampus (HC) are positive, whereas glial cell bodies in the corpus callosum (CC) and the cerebral cortex are negative. *d*, Frontal section of C57BL/6J embryo at 14.5 days *post coitum* hybridized with a *dilute* cDNA probe (nucleotides 3,581–4,217, Fig. 1). The trigeminal ganglion (tg), spinal cord (sc), dorsal root ganglia (drg), brain (b), and skin exhibit higher levels of hybridization than the remainder of the embryo.

METHODS. *a, b, c*, Gel-purified DNA probes were labelled with ³⁵S by nick translation and hybridized to 10-µm frozen brain sections by the method of Haase et al.³¹. Slides were exposed for 28 days before development. *d*, ³⁵S-labelled antisense RNA was synthesized from cDNA template and hybridized to paraffin sections as described by Martin-Zanca et al.³². Slides were exposed for 21 days before development.



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ACKNOWLEDGEMENTS. We thank D. Swing for technical assistance, L. Brubaker for typing the manuscript, and D. Cleveland, A. Peterson, E. Keshet, and members of the Mammalian Genetics Laboratory for reviewing the manuscript. J.A.M. is a Leukemia Society of America Fellow. This research was sponsored by the National Cancer Institute.

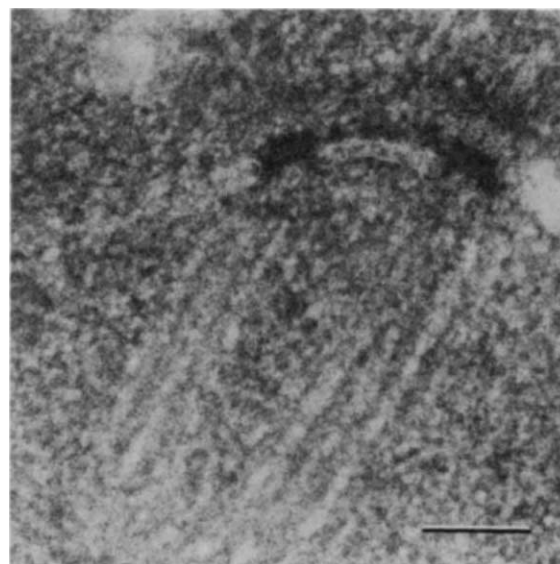


FIG. 1 An electron micrograph of a thin section of a *spo15* cell after 14 h in sporulation medium. Note the duplication of the spindle pole body. Cells were grown to mid-logarithmic phase in YPA medium (1% potassium acetate, 2% bacto-peptone, 1% yeast extract) and transferred to sporulation medium (1% potassium acetate). Cells were prepared for electron microscopy as described by Byers and Goetsch¹¹. Bar, 0.1 μ m.

A dynamin-like protein encoded by the yeast sporulation gene *SPO15*

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THE tightly centromere-linked gene *SPO15* is essential for meiotic cell division in the yeast *Saccharomyces cerevisiae*. Diploid cells without the intact *SPO15* gene product are able to complete premeiotic DNA synthesis and genetic recombination, but are unable to traverse the division cycles. Electron microscopy of blocked cells reveals a duplicated but unseparated spindle-pole body. Thus cells are unable to form a bipolar spindle. Sequence analysis of *SPO15* DNA reveals an open reading frame that predicts a protein of 704 amino acids. This protein is identical to *VPS1*, a gene involved in vacuolar protein sorting in yeast which has significant sequence homology (45% overall, 66% over 300 amino acids) to the microtubule bundling-protein, dynamin. The *SPO15* gene product expressed in *Escherichia coli* can be affinity-purified with microtubules. *SPO15* encodes a protein that is likely to be involved in a microtubule-dependent process required for the timely separation of spindle-pole bodies in meiosis.

The *SPO15* gene is located 200 base pairs from the centromere on the left arm of chromosome XI (ref. 1). Homozygous disruption mutations of the *SPO15* coding sequence result in an early block in meiosis, the terminal phenotype being a mononucleated cell¹. Mutant cells respond to the appropriate nutritional cues, complete DNA replication and recombination with wild-type efficiency, and upon return to a rich carbon source (glucose) re-enter the mitotic cycle. Thus *spo15::HIS3* mutants execute the transition to recombination, but arrest before the meiotic divisions.

The sequence of the *SPO15* gene was determined by the dideoxy method. Sequence analysis predicts a 704-amino-acid open reading frame with a relative molecular mass of 78,687 (EMBL accession number X54316). The DNA sequence is homologous (99.6% at the amino-acid level) to a recently sequenced gene, *VPS1* (ref. 2), identified in a protein-sorting

screen for secretion of carboxypeptidase Y (ref. 3). The sequence of *SPO15* differs by 3 out of 704 amino acids from *VPS1*. Two amino-acid differences result from single-base changes in the nucleotide sequence (*VPS1*: Asn 33 (AAT), Lys 141 (AAG); *SPO15*: Thr 33 (ACT), Gln 141 (CAG)). There is a discrepancy between the nucleotide sequence encoding Asn 111 which is GAA in *VPS1* and AAC in *SPO15*. The *VPS1* gene has been genetically mapped, and is identical to *SPO15*. Complete deletion of *SPO15/VPS1* uncovers a temperature-sensitive process, making cells unable to grow mitotically at elevated temperature². The mutant we describe carries a disruption (*spo15::HIS3*), which leaves the first two-thirds of the gene intact¹; the mutant shows mitotic growth at 37 °C. Differences in scoring temperature-sensitive mitotic lethality may arise from the different nature of the mutations being examined. As described by Rothman *et al.*² the predicted protein product shows homology to interferon-induced Mx proteins and has a consensus sequence for GTP binding. More recently, the sequence of the microtubule-bundling protein dynamin has been determined. This exhibits 45% overall identity to *VPS1* and is 66% identical to *VPS1* over approximately 300 amino acids spanning the GTP-binding consensus sites⁴.

To define precisely the *spo15::HIS3* defect in meiosis, the terminal phenotype has been examined by immunofluorescence and electron microscopy. In *spo15::HIS3* mutants, a meiotic spindle never develops, and the tubulin immunofluorescence remains a diffusely staining spot in thousands of cells examined. Electron microscopy at 14 h following induction of sporulation reveals mostly single or duplicated but unseparated spindle-pole bodies (Fig. 1). Thus the terminal phenotype of *spo15::HIS3* is arrest in the pachytene stage of meiosis. This defect could reflect the perturbation of a regulatory step governing spindle development through the misrouting of vacuolar proteins, or the loss of a direct interaction between the *SPO15* gene product and spindle components.

Microtubules are the major spindle components and are required for separation of spindle-pole bodies in mitosis⁵. The homology of *SPO15* to dynamin is certainly indicative of the potential for microtubule binding. To test the ability of the *SPO15* protein to interact with microtubules, *SPO15* was

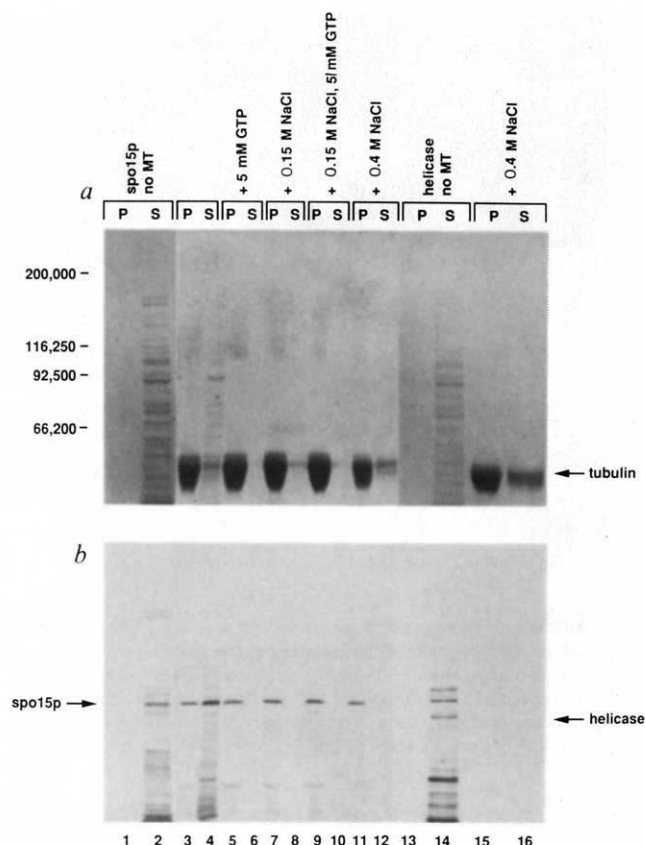


FIG. 2 Expression and microtubule-binding of spo15. *a*, Coomassie blue-stained 7.5% SDS-polyacrylamide gel showing the microtubule-dependent sedimentation of spo15 and helicase II produced from *E. coli* cells carrying the appropriate T7-7 vector. *b*, Corresponding autoradiograph of ^{35}S -labelled proteins. A fraction of the labelled spo15 binds to microtubules and remains bound in the presence of nucleotide triphosphates. Molecular weight markers are indicated to the left. Lanes 1 and 2, pellet (p) and supernatant (s) of the *E. coli* extract (pT7-7 SPO15) in buffer containing taxol in the absence of microtubules (no MT); lanes 3 and 4, *E. coli* extract (pT7-7 SPO15) plus microtubules; lanes 5 and 6, pellet in lane 3 incubated with 5 mM GTP; lanes 7 and 8, *E. coli* extract (pT7-7 SPO15) plus microtubules, bound in the presence of 150 mM NaCl; lanes 9 and 10, pellet in lane 7 incubated with 5 mM GTP and 150 mM NaCl; lanes 11 and 12, *E. coli* extract (pT7-7 SPO15) from a microtubule-pellet fraction incubated with 0.4 M NaCl; lanes 13 and 14, *E. coli* extracts containing pH12-2 (helicase II) in buffer containing taxol in the absence of microtubules; lanes 15 and 16, pH12-2 extracts sedimented with microtubules as in lanes 11 and 12.

METHODS. A 2.4-kilobase (kb) DNA fragment, starting 3 base pairs (bp) upstream of the initiation codon AUG, was inserted 8 bp downstream of the ribosome binding site in the T7 polymerase expression vector pT7-7 (ref. 6), and the junction verified by DNA sequencing. The resulting plasmid (pT7-7 SPO15) was introduced into the *E. coli* strain K38 (HfrC λ) harbouring a plasmid pGp1-2 and labelled *E. coli* extracts were prepared as described⁶. The microtubule-sedimentation assay of proteins expressed from pT7-7 SPO15 was performed with taxol-stabilized microtubules. Porcine-brain tubulin purified on a phosphocellulose column (2 mg ml⁻¹) was assembled into microtubules by incubation at 37 °C for 5 min followed by 20 min at 30 °C in 1 $\times$ PME (0.1 M PIPES, pH 6.9, 5 mM magnesium acetate, 5 mM EGTA, 1 mM DTT) with 1 mM GTP, 20 μM taxol and protease inhibitors. The microtubules were sedimented at 69,000 $\times g$ at 30 °C for 10 min. The pellet was washed twice in 1 $\times$ PME with 20 μM taxol plus protease inhibitors. ^{35}S -labelled proteins treated with apryase (2.5 U per ml) in 1 $\times$ PME with 20 μM taxol were mixed with microtubules and incubated at room temperature for 20 min, then centrifuged through a 10% sucrose cushion (1 $\times$ PME, 20 μM taxol, with protease inhibitors) at 50,000 $\times g$ in a swinging-bucket rotor at 30 °C for 30 min. The supernatants were removed and the pellets washed once in 1 $\times$ PME with 20 μM taxol plus protease inhibitors. The nucleotide-sensitivity and salt-extraction properties of spo15 binding were tested by addition of the appropriate concentration of nucleotide (ATP or GTP) or NaCl to the buffers after the sucrose-cushion sedimentation and incubating the pellet at room temperature for 15 min before the washes.

expressed in *E. coli*. SPO15 coding sequences were placed downstream from a promoter for T7 RNA polymerase and introduced into *E. coli* cells containing a regulated bacteriophage T7 RNA polymerase⁶. SPO15 protein (spo15) was labelled selectively following induction of T7 RNA polymerase in the presence of ^{35}S -containing amino acids. Taxol-stabilized porcine brain microtubules were incubated with clarified ^{35}S -labelled *E. coli* extracts in the presence or absence of nucleotides, and sedimented through a sucrose cushion. The soluble proteins and microtubule-containing pellets were examined by PAGE and autoradiography (Fig. 2). The *E. coli* extract contains a predominant labelled protein of apparent relative molecular mass 85,000. The presence of this protein is completely dependent on SPO15 coding information in the vector. The size estimated from the migration relative to molecular mass standards is in close agreement to that of the predicted open reading frame. Inspection of the autoradiograph in Fig. 2 reveals a significant fraction (>40%) of this protein to cosediment with the microtubule pellet, in the presence or absence of 150 mM NaCl. Incubation of extracts containing the *E. coli* protein, helicase II labelled and prepared in the same fashion, does not reveal significant microtubule-dependent sedimentation. No spo15 was observed to sediment in the absence of polymerized microtubules. Other proteins in the preparation, as visualized by Coomassie- or silver-staining methods, do not sediment with microtubules. In addition, microtubule-bound spo15 is not released by incubation with 5 mM GTP plus 150 mM NaCl, 10 mM ATP (data not shown) or 0.4 M NaCl. Thus spo15, in the absence of other eukaryotic proteins, binds but does not release microtubules in the presence of adenosine or guanosine nucleotide triphosphates, and is resistant to 1 M NaCl extraction (data not shown). This result is of particular interest when considering the bundling function of the related protein dynamin⁷. The ability of dynamin to cross-link microtubules has led to the notion of two microtubule-binding domains⁷. The conserved GTP-binding site may be one, with the other elsewhere in the protein. These may reflect nucleotide-sensitive and -insensitive binding sites, respectively. Alternatively, in the absence of any cellular factors these proteins may not be able to convert from a GTP- to GDP-bound form. Many G proteins are associated with activating proteins required for regulation of GTP hydrolysis⁸, and the mechanochemical activity of dynamin does require the presence of a cofactor⁷. The identification of activating molecules for spo15 will be an important step in determining the role of this new class of microtubule binding-protein in meiosis.

The role of these related proteins (spo15/vps1, Mx, dynamin) in seemingly diverse processes may reflect the multifunctional roles of cellular microtubules. In the case of spo15/vps1, the meiotic role may involve microtubule sliding mechanisms required for spindle-pole body separation, or post-translational regulation of proteins involved in spindle morphogenesis. The requirement for microtubule-binding proteins in spindle function has been recently demonstrated by analysis of KAR3 in bakers' yeast⁹, and bimC4 in *Aspergillus nidulans*¹⁰. These proteins have homology to the microtubule-motor protein, kinesin, and bimC4 mutants arrest with duplicated, but unseparated spindle-pole bodies. The role of dynamin-like proteins in similar processes emphasizes the pivotal role of microtubules and associated molecules in intracellular sorting and spindle morphogenesis. □

Received 30 October; accepted 18 December 1990.

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ACKNOWLEDGEMENTS. We thank D. Fowlkes for assistance in computer analysis of the *SP015* sequence and oligonucleotide synthesis, J. George for advice on *E. coli* protein expression, T. Salmon and his laboratory for tubulin and taxol, and for help with microtubule sedimentation assays. This work was supported by the National Institutes of Health. K.B. was supported in part by a Research Career Development Award from the National Cancer Institute. M.C. was supported in part by a CIDA.

A suppressor of a yeast splicing mutation (*prp8-1*) encodes a putative ATP-dependent RNA helicase

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FIVE small nuclear RNAs (snRNAs) are required for nuclear pre-messenger RNA splicing: U1, U2, U4, U5 and U6^{1,2}. The yeast U1 and U2 snRNAs base-pair to the 5' splice site and branch-point sequences of introns respectively¹. The role of the U5 and U4/U6 small nuclear ribonucleoprotein particles (snRNPs) in splicing is not clear, though a catalytic role for the U6 snRNA has been proposed³. Less is known about yeast splicing factors, but the availability of genetic techniques in *Saccharomyces cerevisiae* has led to the identification of mutants deficient in nuclear pre-mRNA splicing (*prp2-prp27*)^{4,5}. Several *PRP* genes have now been cloned and their protein products characterized. The *PRP8* protein is a component of the U5 snRNP and associates with the U4/U6 snRNAs/snRNP to form a multi-snRNP particle believed to be important for spliceosome assembly⁶. We have isolated extragenic suppressors of the *prp8-1* mutation of *S. cerevisiae* and present here the preliminary characterization of one of these suppressors, *spp81*. The predicted amino-acid sequence of the *SPP81* protein shows extensive similarity to a recently identified family of proteins thought to possess ATP-dependent RNA helicase activity. The possible role of this putative helicase in nuclear pre-mRNA splicing is discussed.

To identify genes encoding splicing factors that interact with the *PRP8* protein, we isolated extragenic suppressors of a *prp8-1* mutation. We sought suppressors that can not only suppress a *prp8-1* mutation but also render the growth of the cells cold-sensitive, as this would greatly aid the analysis of the mutants. Eleven such mutants were isolated which define three complementation groups termed *spp81*, *spp82* and *spp83* (Table 1). Tetrad analysis showed that the cold-sensitive phenotype and the ability to suppress a *prp8-1* mutation were genetically linked in each case and that the cold-sensitive mutations were unlinked to the *prp8-1* mutation and segregated as single recessive nuclear mutations (data not shown). Nine (at least seven of which were independent isolates) of the eleven mutations defined the *SPP81* gene. The ability of the *spp81-3* mutation to suppress the splicing defect of a *prp8-1* temperature-sensitive mutant was confirmed at the molecular level by examining the amount of precursor RNA accumulation at various temperatures. Compared with that observed for the *prp8-1* mutant SPJ8.31, the strain DJY65 (*prp8-1 spp81-3*) failed to accumulate significant amounts of precursor RNA at 34 °C (Fig. 1).

In the process of determining whether the suppressor mu-

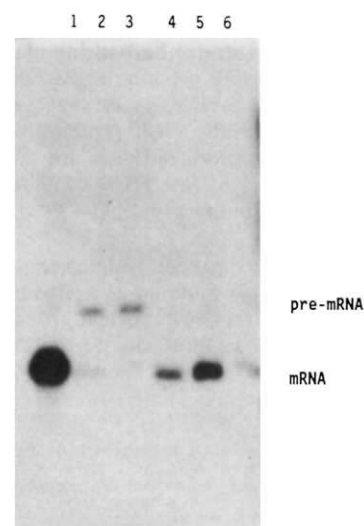


FIG. 1 Northern blot analysis of the *prp8-1* suppressor mutant DJY65 (*MATa his3 leu2 ura3-52 prp8-1 spp81-3*). The yeast actin gene, which contains one intron²¹, was used as a probe to demonstrate suppression of the *prp8-1* temperature-sensitive mutation by the *spp81-3* mutation. Lanes 1-3, SPJ8.31 (*prp8-1*) 23 °C, 34 °C and 36 °C; lanes 4-6, DJY65 (*prp8-1 spp81-3*) 23 °C, 34 °C and 36 °C.

METHODS. The actin-encoding plasmid pYA301 (ref. 21) was labelled by the random-priming method²². RNA was prepared from cultures which had been grown at 30 °C before shifting them to either 23 °C, 34 °C, or 36 °C for 2 h as described by Jackson *et al.*⁷. Total RNA was extracted as described by Hopper *et al.*²³ and equivalent amounts of total RNA were run on a 1.2% agarose gel. Northern-blotting prehybridization, hybridization and washing conditions were as described by Jackson *et al.*⁷.

tations were linked to the *PRP8* gene, plasmids carrying the wild-type *PRP8* gene and flanking DNA were introduced into *spp81* mutants. The *spp81-3* mutants grew very slowly when transformed with plasmid p8000 (2 μ *URA3 PRP8*) compared with those transformed with the control plasmid, YEP24 (2 μ *URA3*). With *spp81-2* mutants the growth defect was more pronounced (no transformants could be obtained), whereas wild-type yeast, *spp82* mutants and *spp83* mutants transformed with plasmid p8000 did not give this phenotype (Table 1). The effect was specific to the *PRP8* gene and was not observed with 2 μ plasmids carrying the wild-type *PRP2*, *PRP4* or *PRP11* genes. Transformation of both *spp81-2* and *spp81-3* mutants with either high (p8000) or low (p8500, *CEN URA3 PRP8*) copy-number plasmids carrying the wild-type *PRP8* gene

TABLE 1 Effect of extra copies of the *PRP8* gene on *spp81* mutants

Relevant genotype	No. of isolates	<i>CEN PRP8</i> p8500	2 μ <i>PRP8</i> p8000
<i>PRP8 spp81-2</i>	9	+/-	-
<i>PRP8 spp82-1</i>	1	+	+
<i>PRP8 spp83-1</i>	1	+	+

Cultures of strain SPJ8.31 (*MATa ura3-52 his3 leu2 prp8-1*) were mutagenized with ultraviolet light to a level at which roughly 90% of cells were killed. Mutagenized cells were divided into aliquots and grown in the dark at 30 °C for 2 days. Aliquots of these pools were spread on YPD plates²⁷ and incubated at 34 °C. Putative cold-sensitive suppressors were identified by screening for weak or no growth at 18 °C and 15 °C on YPD plates. Putative suppressor mutations were then crossed to the wild-type strain KY118 (*MATa ura3-52 trp1-289 lys2-801^{am} adel-101^{oc} his3 Δ 200*)²⁸ and then crossed into the parental strain SPJ8.31 as described by Sherman *et al.*²⁷. Complementation analysis was performed as described by Sherman *et al.*²⁷. To test for interactions with the wild-type *PRP8* gene yeast strains were transformed with *PRP8*-containing plasmids as described by Ito *et al.*²⁹ and plated out at 30 °C. +, growth; +/-, poor growth; -, no growth.

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revealed that the growth defect was related to the copy number of the *PRP8* gene. Yeast strains harbouring plasmids p8000 or p8500 overproduce the PRP8 protein roughly fourfold and twofold, respectively⁷. These results suggest that the product of the *SPP81* gene interacts with the PRP8 protein. It is possible that the excess PRP8 protein produced does not assemble into the U5 snRNP and this pool of free PRP8 protein can effectively sequester a limiting amount of mutant *SPP81* protein into a functionally inactive complex.

A suppressor (*SRN1*) has been isolated that suppresses the defects of several of the *prp* mutants, as well as that of the *rna1-1* mutation which is not defective in nuclear pre-mRNA splicing⁸. The molecular basis of suppression by the dominant *SRN1*

suppressor is unclear, but in view of the nonspecific nature of its suppression, it is probable that the *SRN1* gene product suppresses the defects indirectly. We therefore determined whether the *spp81* suppressors were more specific than the *SRN1* suppressor. The strain DJY63 (*spp81-3*) was crossed to strains harbouring an *rna1-1*, *prp2-1*, *prp3-1*, *prp4-1* or *prp11-1* mutation, and tetrads from each cross were analysed. In no case did *spp81-3* suppress the temperature-sensitive defects of these *prp* and *rna1* mutations (data not shown). These results suggest that the *spp81* mutations are unlikely to be alleles of the *SRN1* gene and imply that the suppression is more specific than that observed with the *SRN1* mutation.

Genetic analysis of the *spp81-2* and *spp81-3* mutations showed that they are tightly linked to the *HIS3* gene on chromosome XV. Two genes of unknown function, *PET56* and *DED1*, have been isolated previously by virtue of their proximity to *HIS3*⁹. To determine whether the cold-sensitive defect of *spp81* mutants could be complemented by DNA from the *HIS3* region, strains DJY57 (*spp81-2 ura3-52*) and DJY63 (*spp81-3 ura3-52*) were transformed with plasmids pYDJ35 (*CEN URA3*) and pYDJ36 (*2μ URA3*) which carry to *HIS3*, *DED1* and *PET56* genes (the 6.1-kb *EcoRI-SalI* fragment from YIP1 (refs 10, 11)). Both plasmids were capable of complementing the cold-sensitive growth defect of *spp81* mutants. Subcloning experiments showed that the 2.8-kb *XhoI-SalI* fragment from YIP1 (ref. 10) could also complement the mutations in both high and low copy-number plasmids (data not shown). As this fragment encodes *DED1*, an essential gene of previously unknown function⁹, the results suggested that the *spp81* mutations might be alleles of *DED1*. To test this, a DNA fragment containing *DED1* was examined for its ability to gene-convert the *spp81* locus in a *spp81-3* mutant, and thereby allow the strain to grow at low temperature. The 2.8-kb *XhoI-SalI* fragment was gel-purified and transformed with pFL38 (*CEN URA3*) into the cold-sensitive strain DJY63 (*spp81-3*). The resulting transformants were analysed for their ability to grow at the nonpermissive temperature. Of the 32 *Ura*⁺ transformants screened, four had acquired the ability to grow at 18°C by gene-conversion, thus demonstrating that the *spp81* mutations are indeed alleles of *DED1*.

The complete nucleotide sequence of the *DED1* gene has been determined (Fig. 2) and indicates that this gene encodes a protein of approximate relative molecular mass 65,000 (65K). Antibodies were raised against the product of a *trpE::DED1* fusion gene containing the C-terminal 492 amino acids of *DED1* and affinity-purified against the product of a *lacZ::DED1* fusion gene containing the same *DED1* fragment. On western blots of total yeast proteins, the antibodies revealed a single band of ~70K; this band was several-fold more intense in strains carrying a high copy-number *DED1* plasmid (data not shown). The predicted *DED1* protein sequence showed no similarity to keratins or other intermediate-filament proteins beyond the small region near the N terminus as previously noted by Struhl⁹. But the predicted *DED1* amino-acid sequence showed strong similarity to a recently identified family of proteins which are thought to possess ATP-dependent RNA helicase activity (Fig. 3)¹²⁻¹⁵. The *DED1* protein is particularly similar to the mouse PL10 protein¹⁶ (62% identical and 78% similar over the highly conserved region). Over the entire length of the proteins, the *DED1* protein shows 49% identity, and 69% similarity to the PL10 protein (Fig. 3). These figures are significantly higher than those for other members of this family¹⁶, raising the possibility that the *DED1* protein is the yeast homologue of the PL10 protein.

The identification of a putative RNA helicase associated with RNA splicing is of considerable interest as the requirement for RNA helicase activity seems to be a general feature of RNA splicing mechanisms. For example, a putative RNA helicase has been identified that is involved in the splicing of some group I and II mitochondrial introns¹⁷. The ATP-dependence of the

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1 CTGAGAAAAAATAAAGAGATGGAGAACGGGAAAAAGTTAGTTGGTGATAGTGGCAA
67 GTGGTATTCCGTAAGAACAACAAGAAAGCATTCATATATGGCTGAACAGCAACAGTCA
    M A E L S E Q V Q
133 AAATTTAAGCATCAACGACACACAGAGATGGTTATGTTCTCTCACTTAAGAGGAAACCAAG
    N L S I N D N N E N G Y V P P H L R G K P R
199 AAGTCCAGAAATAACAGTAGCACTACATAACACACGGCGGTACACCGGTGGCGGTGGCGG
    S A R N N S Y N N N N G G Y N G G R G G
265 TGGCAGCTCTTTAGCAACAACCGTGGTGGTTACGGCAACGGTGGTTCTCGGTGAAACAA
    G S F F S N N R R G G Y N G G F F G G N N
331 CGGTGGCAGCAGATCAACCGCGTCTTGGTGGTAGATGGATGGCAACATGTCCAGCTCC
    G G S R S N G R S G G R W I D G K H V P A P
397 AAGAAACGAAAGCGGAGATGCCATATTTGGTGGTCCCGAGGATCCAAATTCGAATCTTCGG
    R N E K A E I A I F G V P E D P N F Q S S G
463 TATTAAGTTCGATACGATGATATTCAGGAGCGCTCTGGTAAGGATGTCTGAACCAAT
    I N F D N Y D D I P V D A S G K D V P E P I
529 CACAGAAATTAACCTCACTGAGGATGTATTTGGAAACATCAATTTGGCCGTTTCTAC
    T E F T S P P L D G L L L E N I K L A R F T
595 CAAGCCACACCTGTGCAAAATACCTCGCTCCATGCTTGGCAACGGCAGAGTTTGAATGGCTG
    H F T P V Q K Y S V E I V A N G R D L M A C
661 TGGCAGACCGGTTCTGTAAGACTGGTGGTGTATTCAGCTGTGTCCGAATTAAGAC
    A Q T G S G K T G G F L F P V L S E S F K T
727 TGAACATCTCTCAACAGAGCTCAAGGCTCTTTTACCAAGAAAGCGCTACCAACTGCTGT
    G P S P Q P E S Q G S F Y Q R K A Y P T A V
793 CATTATGGCTCAACTAGAGAGTGGCCACCAATTTTCGATGAAGCAAGAAATTTACTTATAG
    I M A P T R E L A T Q I F D E A K K F T Y R
859 ATCTGGGTCAAGGCTGGCTGCTGACGGTGTCTCCAAATGGTAACCACTAAGAGAAATGA
    S W V K A C V V Y G G S P I G N Q L R E I E
925 ACGTGGTGGCATTCTTTAGTCGCTACTCAGGCTGTTGAATGACTTGTGGAACGCTGTAAT
    R G C D L L V A T P G R L D L L E R G K I
991 TTCTTTGGCAACGTCAGATTTGGTGTAGATGAAGTGATAGAATGTGGATATGGGTTTGA
    S L A N V K Y L V L D E A D R M L D M G F E
1057 ACCTCAATAGACATATTTGTCGAGATGTGATGACCTCTGGTGGTAAGACAACTCTGAT
    P O I R H I V E D C D M T P V G E R Q T L M
1123 GTTCACGCTATTTCCCGCTGATATCCAACTATGGCCGCTGATTCTTAAGTGCATACATCT
    F S A T F P A D I Q H L A R D F L S D Y I F
1189 TTTGCTGTTGGTAGAGTCGGTCTACTTCAAGAACTATTCAAAAGCTTTATAGCTGAAAA
    L S V G R G T G S T S E N I T Q K V L Y V E N
1255 TCAAGATAAAGATCAGCTTATTTGATCTATTGCTGCATCCACTGACGGTTTACGTTTATCTT
    Q D K K S A L L D L S A S T D G L T L I F
1321 TGTGCAACTAAGAGATGGCAGATCAATGACCGATTCTGATCATGCAAACTTTAGAGCTAC
    V E T K R M A D Q L T D F L I M O N F R A T
1387 CGCATCTATGGTGACCGATCCCAATCTGAGAGAGAACGTCGCTGGCCGCTTCAGATCTGGTGC
    A I H G D R T Q S E R E R A L A A F R S G A
1453 CGCTACTTATTTGGTGGCAGCTGTCGACGTAGAGTCTAGATATTCCAAGCTGACCCACGT
    A T L L V A T A V A A R G L D I P N V T H V
1519 TATCACTAGATTTACCAAGTATGATGATGATGATGATGATGATGATGATGATGATGATGATG
    I N Y D L P S D V D D Y V H R I G R T G R A
1585 CGGTAAACCGCTTGGCAGCTGCTTCAACAGTGAACACAGTAACTGTAAGGTTTTCGA
    G N T G L A T A F F N S E N S N I V K G L H
1651 TGAATTTTGAAGTAACTAAGAGAGTCCATCTTGAAGGACGCTATGATGAGTGCCTCC
    E I L T E A N Q E V P S F L K D A M M S A P
1717 AGGTAGCAGAAACAGCGTGAAGCGGTTCGCTGCAACACACAGAGATACCGTAAGGC
    G S R S N S R R G G F G R N N N R D Y R K A
1783 CGAGGCGCTAGCGAGCGCTGGGCTCTTCAAGACAGAGATACTCTTTCAGAGCGGTAG
    G A S A G G W G S R S R D N S F R G G S
1849 TGCGTGGGTAGCGATTCAAGCTCTTGGCTGGGTGACAGCGGTGGTTCAACAACTCTTTC
    G W G S D S G S G S G S G S N N S S
1915 GTGGTGATTTCAGACAACTAGGCTGAGGATCTCTGTTTCTGCTTAGCATGCTTATATTTTC
    W
1981 TTCTCCAATCACTTTTTTATCAAGCTCTGAAATACGTGTTGTTGTTTCTCTCTACCGGT
2047 GATTGGCCCATATTTTTTATTTTCTCACTGGGAGCCAACTCTGTTACTTTTTTCCACTCT
2113 TATTTTATCCATCGTTTGTGTTTTTTTCTTTCATGTTCTAATATATTTTACAATTG
2179 ACTATAAATGCCTTTCCTAATATTAACTAAAAAATAAGATTTCACATGATTAAGCAATTA

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FIG. 2 Nucleotide sequence of the *SPP81/DED1* gene and the predicted amino-acid sequence of the *SPP81/DED1* protein. The first 444 nucleotides of the sequence shown are identical to the sequence previously determined by Struhl⁹ except for GT instead of TG at positions 216–217. The one long open reading frame continues that noted by Struhl⁹ and its identity as *SPP81/DED1* is supported by the transcript mapping data reported by Struhl⁹. No consensus splice site signals were found within or near this open reading frame, suggesting that it comprises the complete coding region, as supported also by the size of the protein identified on western blots (see text). This sequence plus an additional 612 nucleotides (to the *SalI* site at the 3' end) have been deposited with the EMBL, Genbank and DDBJ nucleotide sequence data bases.

METHODS. The complete sequence of both strands of the 2.8-kb *XhoI-SalI* fragment was determined using Sequenase (USB) after generating a set of nested deletions in M13 vectors using a modification²⁴ of the method of Henikoff²⁵.

plementary DNA isolated originally¹ was used to rescreen a *Xenopus* oocyte library to isolate full length clones. No single clone spanned the whole of the An3 mRNA, but several contained overlapping regions which allowed the open reading frame of the An3 mRNA to be identified. Analysis of the 5'-most region of the sequence was made possible by polymerase chain reaction (PCR) amplification of the *Xenopus* oocyte library using a sequence near the 5' end of one of the cDNAs already isolated, and a primer that annealed to λ GT10 near the *Eco*RI cloning site⁴.

We have sequenced 4,633 bases of the An3 mRNA and the following features were derived from this analysis. The open reading frame encodes a protein of 697 amino acids with a predicted relative molecular mass of 77,200 (M_r 77.2K). The poly(A) addition site and a portion of the poly(A) tail were present in one of the clones after a 3' untranslated region 2,393 bases long. Preliminary analysis indicated that the difference between the larger and the smaller versions of the An3 mRNA resides in the 3' untranslated region.

The protein encoded by the An3 mRNA was very similar to a protein encoded by a testes-specific mRNA (PL10) found in mouse³ (Fig. 1). The An3 protein contains 697 amino acids compared to 660 for PL10. Seventy-four percent of the amino acids in An3 align with an identical amino acid in PL10, with an additional 4% aligning with a conservative replacement. The greatest variation is in the N-terminal third of the two proteins (Fig. 1). The highest region of conservation, the 393 amino acids between residues 226 and 619, has 94% identity. In addition to the large overall similarity to PL10, An3 protein contains consensus amino-acid sequences found in a family of proteins with helicase or putative helicase activity⁵. This family includes mouse eIF-4A⁶, yeast Tif2⁷ and MSS116⁸, *Drosophila* RM62⁹ and *vasa*^{10,11}, human p68¹², and *Escherichia coli* SrmB¹³ and dbpA¹⁴. Among the several common regions are two motifs often found in ATP-binding proteins. In the An3 protein, the first motif begins at residue 260 (the sequence DLMACAQTGSGKT is a type A-motif) and the second at residue 386 (VLDEADRLDMGF is a type B-motif). These

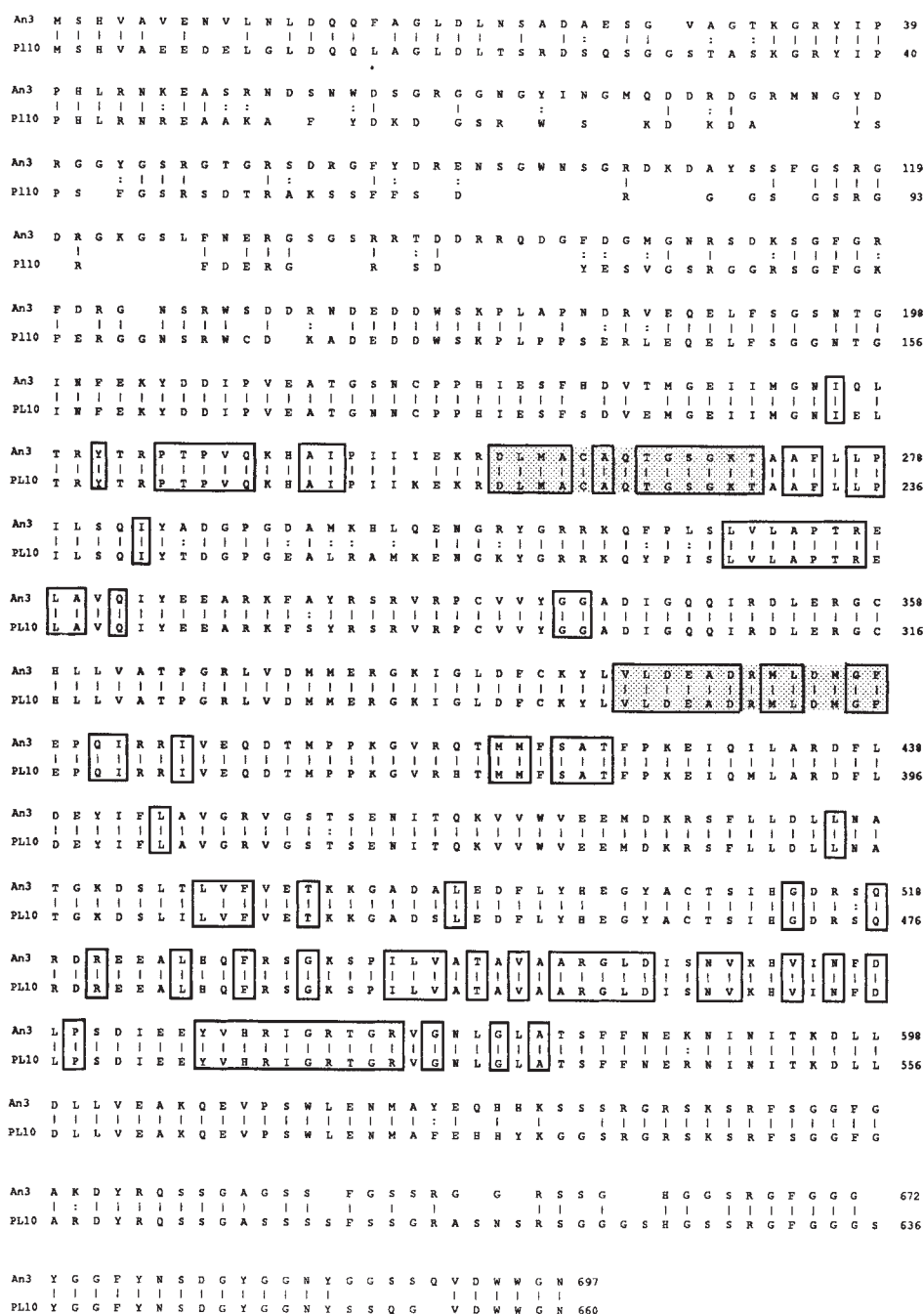


FIG. 1 The amino-acid sequences (single-letter notation) of the An3 protein from *Xenopus laevis* and the PL10 protein from mouse. Boxed regions correspond to regions of consensus sequence among proteins identified as RNA-dependent helicases⁵. Amino acids 260-272 and 386-398, both boxed and shaded, correspond to the motifs commonly found in ATP-binding proteins. Solid vertical lines between the An3 protein sequence and the PL10 protein sequence indicate exact amino-acid matches, broken lines indicate conservative replacements. Sequence analysis using Beckman Microgenie software. The nucleotide sequence of An3 has been submitted to EMBL and has accession number X57328.

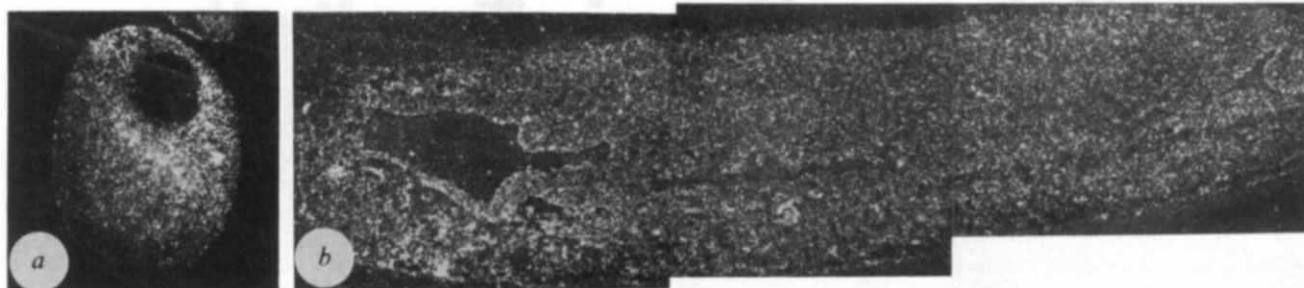


FIG. 2 Distribution of An3 mRNA in *Xenopus* oocyte and embryo. *a*, Localization of the An3 mRNA in a stage VI oocyte²¹. Animal pole of the oocyte is at the top of the figure. *b*, Localization of the An3 mRNA in a stage 22 tadpole²². The orientation of the tadpole is anterior at the left of the panel and dorsal at the bottom. *In situ* protocol and An3 probe was as described previously¹⁵. The complementary hybridization probe was generated by *in vitro* transcription of the An3 cDNA¹ by SP6 polymerase using [³²P]UTP to label the transcript. The pSP65 plasmid containing the An3 cDNA was

linearized with *Hind*III which allowed a runoff transcript of 2.4 kb to be generated. Background levels of hybridization were established using sense transcripts (data not shown). Subtraction of background signal indicates that An3 mRNA is uniformly distributed in the tadpole. Photographs were taken through a Zeiss microscope at a magnification of $\times 100$ using dark field microscopy. The white dots seen in the figure therefore represent the presence of the An3 mRNA in the samples examined.

two motifs are found in a number of different proteins; however, an alanine rather than a glycine in position six of motif A is characteristic of proteins with helicase activity⁵. Other consensus amino-acid sequences are indicated as boxed regions in Fig. 1. These residues may be important for similar functions among the members of the helicase family.

Previous studies have examined the localization of An3 mRNA during oogenesis¹⁵ and embryogenesis through the late blastula stage^{1,2}. Figure 2*a* shows dark field photographs of *in situ* hybridization showing localization of the An3 mRNA in a stage VI oocyte, and illustrates the concentration of this mRNA in the animal hemisphere of the oocyte. Embryonic expression of the gene encoding An3 mRNA begins after gastrulation¹. Unlike the localized distribution found in oocytes and early embryos, upon re-expression An3 is nearly uniformly distributed throughout the embryo (Fig. 2*b*). Although levels of the An3 transcript vary, at least one of the isoforms of An3 is present in most of the adult tissues tested (Fig. 3). Even though the proteins encoded by An3 and PL10 are very similar, An3 mRNA does not exhibit the testes-specific localization of the PL10 transcript described in mouse.

We do not yet know what role An3 mRNA or protein may have in development, but its sequence places it in a family of

proteins proposed to have ATP-dependent RNA helicase activity. The resolution of RNA duplex structure may be important during development for the release of maternal RNA from double-stranded complexes¹⁶, the resolution of double-stranded structure in mRNA during translation (eIF-4A, ref. 25), or the prevention of stable RNA:RNA duplex formation, which has been found to occur in embryos^{17,18}. The unwinding activity first described in *Xenopus* embryos^{17,18} has been found in a variety of tissues and tissue culture cells derived from many species¹⁹. It is interesting that An3 is similarly broadly distributed, although the unwinding activity is currently thought to be ATP independent, and involve RNA modification²⁰.

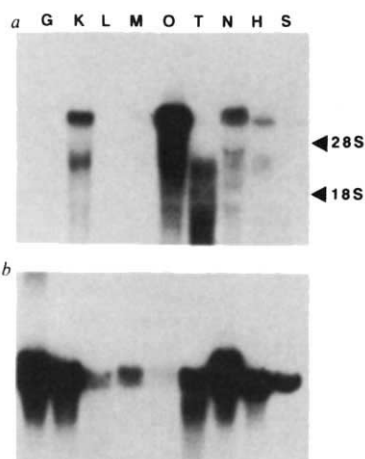
It is noteworthy that two other proteins with similar sequence motifs have been identified in a developmental context. These are the *vasa* protein in *Drosophila* (49% similarity with An3 in the region containing the ATP-binding domain)^{10,11} which is involved in oogenesis and specification of the anterior-posterior axis of the embryo and the testes-specific PL10 protein from mouse³. Although An3 is a localized maternal mRNA in oocytes, eggs and early embryos, later in development it does not exhibit the restricted expression of *vasa* or PL10. Current studies aim at determining when the An3 mRNA is translated, and isolating An3 protein to determine its activities. □

Received 5 November 1990; accepted 2 January 1991.

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ACKNOWLEDGEMENTS. We thank J. Linnen and J. Potts for comments on the manuscript, J. Fassler for assistance with sequence analysis and members of the Walder lab for computer assistance. We also thank P. Krieg for providing the EF-1 α plasmid used in this study. This work was supported by the NIH (D.L.W. and D.A.M.).

FIG. 3 Analysis of An3 mRNA in *Xenopus* tissues. *a*, Levels of An3 mRNA. *b*, EF-1 α mRNA in different tissues. G, adult gut; K, adult kidney; L, adult liver; M, adult muscle; O, oocyte; T, testes; N, adult lung; H, adult heart; S, adult skin. EF-1 α analysis was used as a control for RNA integrity²³ and although its levels also vary in different adult tissues, establishes that undegraded RNA was present in every lane. RNA (20 μ g) from each tissue sample was loaded in each lane of a formaldehyde-agarose gel. After electrophoresis, the RNA was transferred to a Nytran (Schleicher and Schuell) membrane and hybridized with radioactive probes. Random-primed [³²P]dATP probes were generated using either a subfragment derived from the open reading frame of the An3 cDNA (between amino acids 104-272) or the EF-1 α cDNA previously described (ref. 23). RNA was isolated by guanidinium isothiocyanate extraction as described²⁴.



or glycine/hydrophobic/hydrophobic/polar or glycine, whereas the five amino acid pattern is charged or glycine/hydrophobic/hydrophobic/hydrophobic or proline/polar or glycine. The correlation of these patterns with known epitopes has been shown to be statistically significant².

"D" and "d" motifs. These two predictive algorithms are based on the work of Sette *et al.*³. These researchers described sequence motifs that were devised to predict the capacity of peptide molecules to interact with the mouse MHC class II (Ia) molecules, I-A^d (the "D" motif) and I-E^d (the "d" motif). The "D" motif was identified on the basis of structural similarities among unrelated I-A^d binding peptides using a 'master sequence' from a I-A^d binding ovalbumin peptide. The algorithm searches for residues that match the ovalbumin (OVA) sequence, which are structurally similar, or which were found empirically to be tolerated when substituted for the original OVA amino acid. A numerical 'motif number' is obtained, which is based on numerical values that have been assigned to the amino acids. A number greater than a minimal threshold value scores positively as a "D" motif. The "d" motif was derived empirically by comparative analysis of peptide sequences that were known to bind to the I-E^d Ia molecule. Subsequent experiments³ showed that these motifs successfully predicted Ia binding in approximately 75–80 per cent of cases and suggested that they could be of general use in predicting potentially immunogenic peptide regions within proteins.

Software interface

TSites takes advantage of the Macintosh user interface and for input uses a TEXT or ASCII file that contains the amino acid sequence of a protein or peptide in the single-letter code. The maximum number of amino acid residues in one sequence that can be analysed by the program is 10,000. The user may select all or any part of the sequence for analysis.

The software operates on all Macintosh computers from a Macintosh Plus to the most recent models. Use of Macintosh computers with a maths co-processor results in significantly faster analyses. For example, analysis of a 153 amino acid residue sequence, such as sperm whale myoglobin, will take six seconds on a Macintosh SE/30 or two seconds on a Macintosh IIfx. Analysis on a slower Macintosh computer such as the SE or Classic will take 215 seconds. □

David C. Feller and Vidal F. de la Cruz are at MedImmune, Inc., 19 Firstfield Road, Gaithersburg, Maryland 20878, USA. Copies of TSites can be obtained free of charge by sending a 800 K or high-density disk with a self-addressed disk mailer to the above address. Documentation and instructions on

Program guide

Shopping for software? DNA ploidy analysis software, PCR primers by design, and a protein modelling package are a selection of this week's scientific software highlights.

THE California-based company The Lock Box has brought out a new **security device for computers**, imaginatively titled The Lock Box, which is designed to tighten up security with networked Macintosh computers and Macs that are frequently left unattended (*Reader Service No. 101*). The



Locked tight with The Lock Box.

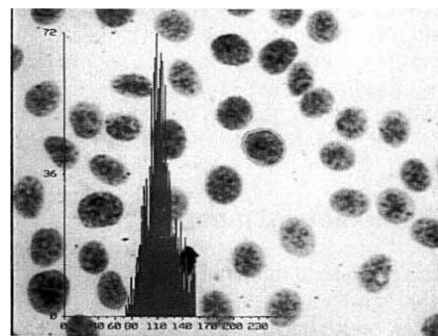
Lock Box provides four main features: manual key lock of mouse and keyboard, autostart of Mac II systems in the event of power failure after the power is restored, remote lock/unlock of mouse and keyboard from a user-provided switch to prevent unauthorized use, and remote start of Mac II systems from a user-provided switch. The \$99 (US) 3 × 3 × 4-inch device opens up like a clamshell to reveal ADB connectors for the keyboard and mouse, and two screw terminal connectors for the remote switches. The box is closed after the user connections have been made. The entire operation takes less than five minutes, says the company.

WinBasic, an **integrated BASIC compiler/editor for Windows applications** brings Windows programming within the grasp of programmers who currently solve laboratory problems in BASIC,

the use of TSites are provided, along with a sample input file and samples of the different types of output files. The program is not copy protected. However, researchers who are interested in obtaining a copy should do so directly from MedImmune, Inc. so that information on program revisions and future updates can be better distributed. For more information, fill in reader service number 100.

Turbo Pascal and dBase, says Blackwell Scientific Software (*Reader Service No. 102*). Before the introduction of WinBasic, Blackwell says programming for Windows would have involved an unenviable choice: write an application from the ground up in 'C' (or assembler), use a specialist high-level Windows language or use macros to customize an existing application. In addition to Windows programming, WinBasic also brings smaller applications, whose development overheads in 'C' would be prohibitive, within the scope of Windows. WinBasic can be used on anything from a humble 640-K AT to the latest 16-MB i486 machine. According to the company, one line of WinBasic code can achieve as much as 50 lines of 'C'.

Dynatech is offering new **DNA ploidy analysis software**, which is designed for use with the company's SAMBA 4000 Cell Image Analysis System, a system that integrates cell image acquisition, analysis and data processing to transform images into repeatable, quantitative measurements (*Reader Service No. 103*). The program measures six morphometric and densitometric parameters of stained



Dynatech's DNA ploidy analysis program.

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nuclei and can be used with any nuclear staining reagents and procedures, says Dynatech. When running the software, the SAMBA System will display and print DNA histograms and automatically calculate DNA, proliferation and aneuploid indices. Programs for immunolabelling, autoradiography and hybridization are also available. New software programs for bone morphometry, nerve morphometry, fluorescence and metaphase finding are available for use in the Open Software package of the SAMBA System.

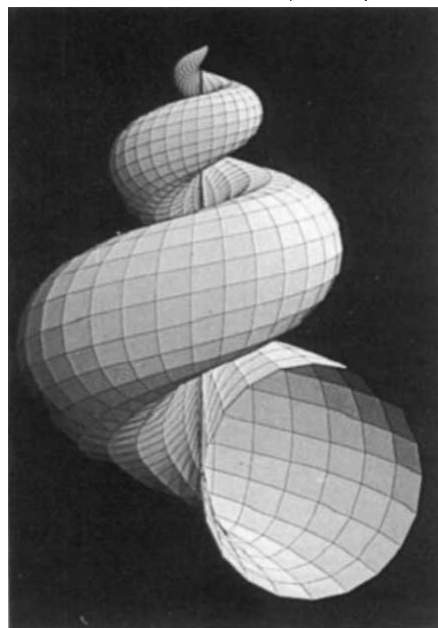
A team of geneticists and computer scientists from the Jackson Laboratory has developed a **software package for genetic research** entitled "Encyclopaedia of the mouse genome", which integrates data that has been compiled by independent researchers (*Reader Service No. 104*). The software has been tailored for geneticists and provides easy-to-use graphic displays, says Jackson Laboratory. To illustrate the versatility of the software, a researcher looking for information on, for example, Lesch-Nyhan syndrome — a rare genetic disease that causes spasticity and self-mutilation — can select the gene responsible for this disease from a list of all mouse genes. The user can then simultaneously call up a separate data base for mouse gene mapping, locate the gene on the X chromosome and find 28 references relating to other studies on this particular gene. Another command allows the user to see what patterns characterize the place on the chromosome where the gene is located and, at the same time, see what other mammals carry the gene in a comparable location. The encyclopaedia is available on diskette or tape at present, but it is hoped that eventually researchers will be able to tap into new data that will be updated though the computer network.

Mathematical means

SolvEq, a general purpose **equation solver and numerical modelling tool** for IBM PC, PS/2 and compatible computers is now available from Biosoft (*Reader Service No. 105*). The program allows the user to enter and solve mixed systems of linear or non-linear algebraic and differential equations. SolvEq supports a modular approach to design, allowing large complex models to be built from smaller reusable components; each of the components can be solved and tested separately, and then merged into the full model, says Biosoft. The SolvEq editor uses familiar notation for entering and displaying equations. Users can type all expressions and operators directly from the keyboard or select from pop-up menus. SolvEq automatically formats special expressions, such as integrals, derivatives and summations, as they are entered. In addition to the usual text-

editing commands, the editor provides a file-merging feature that ensures parameter values and attributes are correctly merged along with the equations. SolvEq sells for £149 (UK), \$299 (US).

Version 2.0 of Mathematica, a **software system for numeric, symbolic and graphical computation** for researchers in disciplines as diverse as physics and engineering to life sciences, will be available from Wolfram Research in early 1991 (*Reader*



Mathematical model of a molluscan seashell using Mathematica 2.0.

Service No. 106). The number of functions in Mathematica 2.0 has increased from 560 to 843. Version 2.0 now provides the ability to solve differential equations, enhanced graphics, better coordination with external programs, international character sets and messages, automatic networking and linear programming. In addition to increased functionality, the performance of the software has been boosted with a compiler that allows complex numerical expressions to be executed up to 20 times faster than in version 1.2, says the company. The programming language has been enhanced to facilitate the programming process. On the Macintosh, NeXT, Sony and Sun computer platforms, which have built-in sound capabilities, users can now take data and functions and render them in audible form, in order to hear as well as see the results. Prices for Mathematica 2.0 vary depending on the platform on which the program runs (with Mathematica 2.0, there are nearly 20 platforms). Prices start at \$595 (US) for the Macintosh and rise to \$30,000 (US) for the CONVEX platform.

Design Science has introduced MathType, a **mathematical equation editor** for IBM PCs or compatibles that works in the Microsoft Windows graphical environ-

ment (*Reader Service No. 107*). Following several years after the release of a version for Macintosh computers, MathType for IBM PCs/compatibles allows the user to build complex equations using point-and-click techniques. There are no command languages or codes to learn, says the company. MathType automatically sizes, spaces, and positions symbols, and uses the correct typeface for standard function abbreviations such as log and sin. Output is produced in PostScript, which allows features such as fractional point sizes and manual character positioning in half-point increments. As MathType operates in the MicroSoft Windows environment, it can be run simultaneously with word processing, desktop publishing, or graphics programs. MathType costs £199 (UK) and is available on 3.5- and 5.25-inch disks.

Designer primers

A **primer design software** package called Primer Detective for IBM PCs or compatibles is now available from Clontech (*Reader Service No. 108*). Primer Detective allows the user to search DNA or RNA sequences for suitable primer sets that are based upon user-specified selection criteria. In this way, primers can be evaluated before they are synthesized for use in sequencing, PCR and other applications, says Clontech. The program's



Primer Detective — for high-quality DNA oligo primers by design.

'advanced' options allow users to find nested and overlapping primer sets, compare primer sets from different sequences and find restriction enzyme sites. Multiple searches can be saved and retrieved and, by using the 'View Sequence' option, users can 'see' the location of all primers within a sequence. DNA or RNA sequences can be imported into Primer Detective from GenBank or ASCII files. Primer Detective, which costs \$295 (US), requires 256 K of memory and is available on either a 3.5- or 5.25-inch disk.

Primer Designer is a new program from Scientific and Educational Software that may be of interest to molecular biologists who routinely select **primers for PCR amplification, sequencing or oligo-directed mutagenesis** (Reader Service No. 109). PCR primers are designed by loading a sequence file and identifying the region to be amplified. The user has control over the length of primers and 5' extensions, if any. The position of the left and right primers can be moved from their starting positions until suitable primers are found. Primers can be moved manually, one position at a time, or they can be moved to the next base position that meets the primer evaluation criteria by using a single keystroke to invoke the automated scan option. The user can set acceptable limits for GC content, melting temperature, degree of permissible secondary structure and the maximum number of identical bases permitted in a run. In addition, different criteria can be set for left and right primers and pairs of PCR primers can be compared to show the presence and extent of primer-primer interactions. Primer Designer can also be used to design oligo primers for use in sequencing or mutagenesis. The software runs on IBM PC/XT, AT, PS/2 or compatible computers. Individual and departmental licences cost \$150 (US) and \$400 (US), respectively.

Molecular models

Biosym Technologies has released version 1.1 of Homology, a **protein model building program** that allows the user to build three-dimensional protein models of a newly characterized protein when knowing only its amino acid sequence and the structure of other related reference proteins (Reader Service No. 110). This latest version of Homology includes automatic as well as interactive determination of 3D structurally conserved regions, secondary structure prediction and hydropathy analysis. Version 1.1 retains parallel 3D structure and sequence alignments, model building of an unknown structure from reference proteins, and refinement of built models through default or user-defined energy minimization and molecular dynamics strategies. Protein models built using Homology can be used in drug design studies or, alternatively, as a starting point for X-ray or NMR structure refinement.

MacImdad 2.0 from the Molecular Applications Group brings **interactive macromolecular graphics** to the desktop Macintosh II computer (Reader Service No. 111). Over 500 protein and nucleic acid X-ray structures can be accessed and drawn, together with any organic molecule that is specified by a general chemical formula, says the company. Sets of coordinates can be read in and converted



Macromolecular graphics for the Mac.

to the compact MacImdad format for rapid display. Molecular drawings in a combination of different styles, including stick bonds, balls and sticks, space-filling atoms and dot surfaces, can be oriented and then animated to give rotating three-dimensional images. MacImdad provides versatile control over the colour of atoms, residues and whole chains, and can display sections through solid models to show the internal packing. Standard DNA double helices of any sequence can be built to facilitate the visualization of possible protein recognition binding sites. Polypeptide chains of arbitrary sequence and conformation can be drawn and manipulated. Multiple molecules can be packed together and the program can automatically superimpose different model or X-ray structures. MacImdad 2.0 runs on Macintosh II computers.

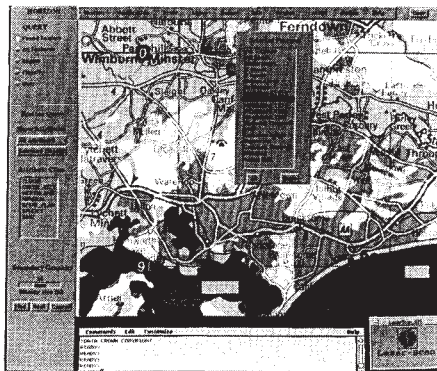
Nemesis, an interactive **molecular modelling package** from Oxford Molecular that has been available on the Macintosh since August 1990, will soon be available for IBM PCs or compatible computers operating under Windows 3.0 (Reader Service No. 112). The structure editor menu of Nemesis provides facilities for building and modifying structures in three dimensions. A library of energy-minimised substructures is supplied with Nemesis. The software allows the user to move quickly between display styles, says Oxford Molecular. Structures may be displayed as stick models in split-bond or single-colour mode, or as space-fill models. Nemesis provides an interface to Connolly's molecular surface program for the generation and display of dot surfaces; these may be coloured by atom type or electrostatic potential. Conformational searches are performed in Nemesis using the COSMIC force field. The conformations menu may also be used to search for conformations that satisfy user-defined geometric constraints. Both the IBM and Macintosh versions of Nemesis cost £500 (UK). Shipments of the IBM version will begin next month.

Applications software

The Aardvark **neuroendocrinology workstation** from Omega Computers represents the collaborative efforts of neuroscientists and computer hardware and software engineers (Reader Service No. 113). The workstation, which is based on

a high-resolution patch clamp amplifier with an in-built phase-lock amplifier, is suitable for the study of secretory cells and for performing all standard measurements of membrane electrical parameters (single-channel and whole-cell recording in voltage clamp and current-clamp modes) on cells grown in culture, cells in brain slices, lipid bilayers and giant phospholipid liposomes. In addition, there is a hardware data acquisition system (IBM PC or compatible and an Aardvark 12-bit, eight-channel, analog-to-digital converter) and software for the real-time estimation of membrane conductance, membrane capacitance and access conductance.

Laser-Scan has launched a Geographical Information System called Horizon that can be used to **predict and assess the effects of change on the natural environment** (Reader Service No. 114). Horizon permits the integration of information such as maps, statistics, socio-economic data, terrain models and satellite imagery. The program provides the user with tools for displaying maps and changing scale.



Horizon: environmentally-friendly software.

Information can be presented in a variety of formats: from basic map data to views of realistic terrain models. This level of detail and power of prediction for 'what if' planning is designed to help planners and land managers ensure that future developments have a minimum impact on the environment, says the company.

Jeol's new **data system for its mass spectrometers**, featuring the Complement software program, is designed to facilitate data processing (Reader Service No. 115). Multi-window displays and mouse-controlled functions simplify the operation of mass spectrometers and ease data reduction, says Jeol. The multi-tasking Complement software provides full control of Jeol's mass spectrometers plus processing for data reduction, quantization, exact mass measurement, selected ion monitoring and system management. The data system uses the 32-bit HP 9000 series of engineering workstations. By virtue of the UNIX-based operating station, the system can also be linked — via ETHERNET — to other instruments,

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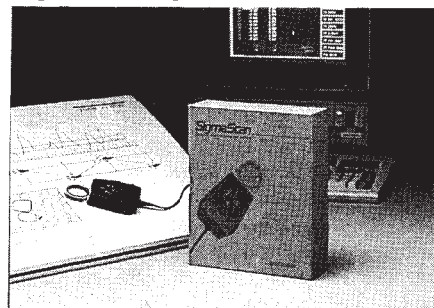
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such as NMRs. The new data system, which is available with all new mass spectrometers purchased from Jeol, can be retrofitted to existing SX, DX, AX and HX instruments.

Measuring up

Vision Toolbox, an image processing software package for Macintosh II computers from Automatix, allows users to extract measurements or other qualitative data from video images (*Reader Service No. 116*). The £450 (UK) Vision Toolbox package permits the capture and manipulation of images, and enables both quantitative and qualitative measurements to be made. Vision Toolbox works with either a frame grabber board and any standard video camera or disk-stored images. All images, data and results can be output directly to PostScript printers. Images can be saved as TIFF or PICT files for use in desktop publishing and graphics applications. For the scientific medical and photographic imager, the interactive tool palette and enhancement mode may be useful. In general, these images will have been acquired previously by other devices and then accessed by Vision Toolbox as PICT or TIFF files. Information can then be extracted by direct inspection and interpretation of the image in grey scale, enhanced, or pseudo-colour.

Rulers and calculators may be a thing of the past with the new SigmaScan scientific measurement system from Jandel Scientific, which can be used in conjunction with IBM PC or compatible computers to digitise, measure and analyse two-dimensional specimens (*Reader Service No. 117*). The user, after placing the specimen on the tablet, selects the type of measurement to be performed, specifies the units and cali-



SigmaScan measurement system lets you digitise, measure and analyse 2-D specimens.

brates. Using the cursor of the digitiser, the user can trace areas, follow lines, define angles and count items. Measurements are transferred automatically into the data worksheet (16,000 columns by 65,000 rows) as they are collected. Data can be saved to disk in ASCII or binary formats. In addition, users can perform data manipulations and transformations, and create keyboard macros to speed individual analyses. The complete Sigma-

Scan scientific measurement system provides the user with a choice of digitising tablet in a variety of sizes — opaque for general purpose and high-accuracy digitising and translucent for measuring specimens that require backlighting.

Unitran, unit conversion software from Intellibase Engineering Software, has over 400 predefined units and physical constants in 75 categories, such as acceleration, kinematic viscosity, although more can be added (*Reader Service No. 118*). Entire tables of data can be imported and converted. In addition, formulas can be generated with parameters in inconsistent units, and Unitran will calculate results in SI, says Intellibase. The \$79.95 (US) Unitran package is menu-driven with on-line help. It runs on PC, PS/2 DOS-compatible hardware and requires 640 K RAM.

Statistical analyses

This month, Cherwell Scientific released C-Stat — an upgraded version of the company's Oxstat statistics package (*Reader Service No. 119*). Using pull-down menus, pop-up dialogue boxes and improved direct data input using spreadsheet commands, C-Stat provides the user with all the basic statistical tests for use on data sets of up to 50,000 data items. A special LogFile feature allows users to keep their analyses as TEXT files on disk. The LogFile can be printed at any time or incorporated into word-processed documents for reports and analyses. Data files can also be exported to graphics programs such as Harvard Graphics, Fig P and EasyPlot. For £55 (UK), researchers can upgrade Oxstat to C-Stat. New copies cost £195 (UK).

CSS: Statistica is the latest statistical analysis and graphics software package from Statsoft UK (*Reader Service No. 120*). Some of features of the £545 (UK) CSS:Statistica package include nonlinear estimation methods, Logit and Probit analysis, ANOVA/ANCOVA/MANOVA/MANCOVA, log-linear analysis, stepwise discrimination function analysis, cluster analysis, multidimensional scaling, time-series techniques with modeling, and polynomial lag analysis. The package also offers a wide selection of two- and three-dimensional graphical displays. Graphs can be displayed on most mono and colour graphics displays, including super VGA and ultra-high resolution graphics co-processor-based systems. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.